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THE INFLUENCE OF CHEMICAL CONSTITUTION ON ANTI-BACTERIAL ACTIVITY. PART III: A STUDY OF 8-HYDROXYQUINOLINE (OXINE) AND RELATED COMPOUNDS.

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Received for publication December 30, 1946.

AT various times hydroxyquinolines have been tested for antibacterial activity (von Oettingen, 1933), but never under conditions where their relative potencies could be assessed. The best known of the seven isomerides is 8-hydroxyquinoline, also known as oxine, and for brevity we propose to use the latter name. The familiar Chinosol of commerce is a mixture of oxine sulphate and potassium sulphate.

Since oxine is known to be active against bacteria and fungi in very high dilution, it probably acts by interfering with particular metabolic processes essential for the growth of these organisms. That this specific action might well take the form of a combination between the drug and trace metals, essential in bacterial metabolism, was put forward by one of us (Albert, 1944). This suggestion was based on the fact that oxine readily *chelates*, that is to say, it combines with polyvalent metals to give feebly dissociated complexes in which the metal forms part of a heterocyclic ring system. Those compounds with groups which are so placed and are of such a nature that they can bind a metal in this manner were termed " chelate " by Morgan and Drew (1920) from analogy with *chela*, the crab's claw.

The discovery that 1-hydroxyacridine has an antibacterial activity greater than could be accounted for on the basis of kationic ionization (Albert, Rubbo, Goldacre, Davey and Stone, 1945) stimulated the present investigation, since it was realized that the hydroxyl group in 1-hydroxyacridine is situated, relative to the nitrogen atom, exactly as in oxine (Fig. 1). Hence it was thought desirable to determine whether the same specificity for the position of this hydroxyl group obtained in the quinoline series as in the acridine series.

Whilst the present studies were proceeding along these lines, Zentmyer (1943) suggested that oxine may act on fungi through chelation with essential metals, and later (1944) he demonstrated a reversal by zinc.

The results of our investigations show that oxine, which is the only monohydroxyquinoline capable of chelation, is the only one of seven possible isomerides

to possess antibacterial properties. Further, it is shown that not only oxine, but also structurally related substances that chelate equally well, are particularly active against Gram-positive types. Whilst chelation is revealed as the essential attribute determining the activity of this series of drugs, several other types of chelate compounds are shown to be without a like antibacterial activity. Thus the architecture of the drug molecule is no less important than its potentialities for chelation.

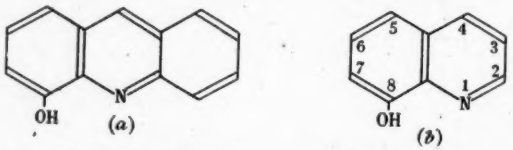

FIG. 1.—(*a*) 1-Hydroxyacridine. (*b*) 8-Hydroxyquinoline (oxine).

An important extension of the present work will be concerned with the nature of the metabolically essential metal or metals that are inactivated by oxine. Preliminary experiments reported here indicate that cobalt may have an importance not previously ascribed to it in nutritional studies of micro-organisms.

CHEMISTRY.

Many metals, in the form of their salts, react with appropriately constituted organic compounds to give ring structures having totally new properties. The chelate complexes thus formed have stabilities which vary, but are often of a high order. Such complexes are usually only sparingly soluble in water and display an increased solubility in organic solvents. Their metallic salt-like nature is masked, and they resemble organic, rather than inorganic, compounds.

The present work is concerned principally with those chelate complexes in which a metal forms a new ring by linking simultaneously with a phenolic oxygen (through a primary valency) and with nitrogen (by co-ordination). For example, two molecules of oxine react with one molecule of a zinc salt to give a chelate complex $Zn(C_9H_6ON)_2$, as in Fig. 2, and in this way an aqueous solution of a zinc salt can be largely deprived of its zinc ions. Oxine also chelates with trivalent metals as in $Fe(C_9H_6ON)_3$; molybdenum and allied metals give complexes of the type $MoO_2(C_9H_6ON)_2$.

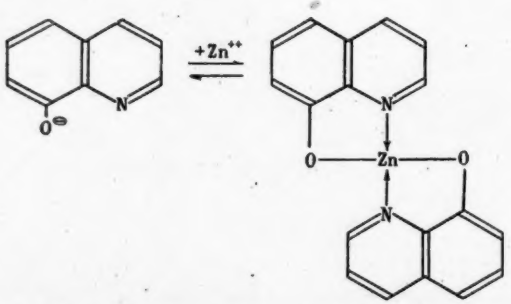

FIG. 2.—Reaction between two oxine anions and one zinc ion to give oxine-metal chelate complex.

As will be seen, oxine chelates with metals to form five-membered rings in which the co-ordinate bond between metal and nitrogen consists of two electrons which are supplied by the latter (this is indicated by the use of an arrow pointing to the metal). Furthermore, it is most likely that the hydroxyl group in oxine must ionize as an anion before it can contribute to the formation of a chelate ring.* Therefore, any factors that interfere with the availability of the electrons from the nitrogen atom or with the ionization of the hydroxyl group will interfere with chelation. Such factors include (I) the saturation of the lone pair of electrons by an alkyl group (as in N-methylation) or by a hydrogen ion (as in internal salt formation), and (II) repression of ionization of the hydroxyl group either totally, by etherification, or partially through the adverse influence of electron-repelling substituents. The influence of these factors on antibacterial activity has been investigated in the present work, and in general the connection between chelation and activity is well maintained.

Although it is intended here to deal principally with the quinoline series, similar hydroxy compounds from related families such as phenazines and phenanthrolines have been included. In addition, a large number of miscellaneous organic reagents used in chemical analysis for the determination of traces of metals have been examined biologically.

In collateral publications (Albert and Gledhill, 1947; Albert and Magrath, 1947) the chelate powers of all these substances are compared by a semi-quantitative technique, using the degree of precipitation as a measure of chelation. This method was especially designed to test chelate strength at the physiological pH of 7·3, so that the bacteriological findings of the present work could be correlated with chelation. The degree of chelation is indicated in the following tables by setting the chemical symbols in two columns. "Strong" connotes a pronounced reading either in the form of a heavy precipitate at standard dilution (M/1000) or as a slight, but definite, precipitate at a tenfold further dilution. "Less Strong" implies a slight but definite precipitate at the standard dilution only. Where the symbol is not cited, no chelation took place under these conditions; for instance, in no case did calcium or magnesium react. The metals investigated were chosen because of their biological interest, namely, calcium, magnesium, manganese, zinc, iron, cadmium, cobalt, lead and copper. Ferrous and ferric iron behaved similarly under aerobic conditions.

It is important to note that the firmness with which a chelate compound can hold a metal varies with the type of chelate groups. Groups of the type now under consideration are known to confer a high degree of stability on the complex. Nevertheless, even these metal complexes exist in equilibrium with small proportions of free metallic ions, a fact which makes reversal possible under carefully controlled conditions. Amino acids present in the medium are also capable of chelation, but it seems likely that the metallic complexes thus formed are metabolized by the bacterium. An exact measure of the equilibrium between each chelate complex and its constituents would be afforded by determination of the *stability constant*:

$$K = \frac{[\text{metal oxine complex}]}{[\text{metal}]\ [\text{oxine}]}$$

Unfortunately no satisfactory method is available.

* The anionic ionization of oxine at 37° C. and pH 7·3 is approximately 1·5 per cent.

The preparation of the compounds used is described in a collateral paper (Albert and Magrath, 1947). For the purpose of the present study every care was taken to exclude traces of heavy metals in the finished product.

BACTERIOLOGY.

The same general technique for testing was followed as in Parts I and II of this series, except that the medium used was a meat infusion broth prepared according to Wright's method (1933), plus 0·05 per cent glucose when using streptococci, and adjusted to pH 7·2–7·4 (glass electrode). Plain broth was chosen in this work in preference to serum broth, because it was anticipated that serum might be an interfering factor in subsequent metal reversal experiments. Serum broth (10 per cent), however, was used for testing the activity of a number of substances of possible clinical interest (Table VIII). No attempt was made to exclude trace elements during preparation of the media.

The broth medium was distributed aseptically by means of an automatic 25 ml. pipette into sterile six-ounce bottles and incubated for 48 hours at 37° C. before use. Each derivative was dissolved in sterile water to give an M/80 solution with the aid of 1·5 equivalents of hydrochloric acid, except where the substances were strongly acidic (–COOH, $-SO_3H$, –Cl and $-NO_2$ derivatives), in which cases 1·5 equivalents of sodium hydroxide were used instead. The first dilution was usually prepared by adding 5 ml. of M/80 solution to 45 ml. of broth, thus giving an initial concentration equal to M/800. Geometrical (twofold) dilutions were made by transferring 25 ml. volumes to each successive dilution bottle. The broth dilutions were then transferred to test-tubes, each bottle yielding twelve 2 ml. quantities, which sufficed to test six organisms in duplicate.

The organisms used in this work were the stock strains used in Parts I and II, plus a pigment-producing strain of *Pseudomonas pyocyanea*. The inoculum, as before, was standardized as 0·03 ml. of a 24-hour culture. The highest dilution which completely prevented visible growth after 48 hours' incubation at 37° C. was taken as the end-point of bacteriostasis. The concentrations required to achieve this result are given in all tables. In comparing two substances, a difference of more than twofold in duplicate tests was considered significant.

RESULTS.

The material has been classified into four categories (Tables I to IV) on the basis of molecular structure.

(1) *Quinoline and the Seven Mono-hydroxyquinolines.*

In this group of compounds (Table I) the strong positive correlation existing between chelating power and antibacterial activity is plainly seen. Of the compounds listed in Table I only the 8-hydroxy isomeride, oxine (No. 8), is capable of chelation, and it alone is antibacterial. The bacteriostatic index against the Gram-positive organisms is especially high. The figures for *Cl. welchii*, *Str. pyogenes* and *Staph. aureus* (M/100,000) correspond to a dilution of one part in 680,000 approximately, a titre considerably higher than the best of the acridine drugs, e.g. 5-aminoacridine (No. 9). This superiority, however, is reversed in favour of the acridines where Gram-negative organisms are concerned.

This series of isomeric compounds is an outstanding example of biological specificity in so far as only one of the seven isomerides manifests definite anti-

TABLE I.—*The Mono-Hydroxyquinolines.*

Highest dilution expressed as $\frac{1}{\text{molarity}}$ of the substance completely preventing visible growth in 48 hours at 37° C.

(Medium : Broth, pH 7·3.)

Number.	Substance.	Criterion of purity.	M.W.	Organisms.						Metals chelated at pH 7·3*.	
				Cl. welchii.	*Str. pyogenes.*	*Staph. aureus.*	*Bact. coli.*	Proteus.	*Ps. pyocyanea.*	Strongly.	Less strongly.
1	Quinoline (for comparison)	b.p. 238° C.	129	—	—	—	—	—	—	None	None
2	2-Hydroxyquinoline	m.p. 199°	145	—	—	—	—	—	—	,,	,,
3	3- ,,	m.p. 198°	145	—	—	—	—	—	—	,,	,,
4	4- ,,	m.p. 200°	145	—	—	—	—	—	—	,,	,,
5	5- ,,	m.p. 223°	145	—	—	—	—	—	—	,,	,,
6	6- ,,	m.p. 193°	145	—	—	—	—	—	—	,,	,,
7	7- ,,	m.p. 235°	145	—	—	—	—	—	—	,,	,,
8	8- ,,	m.p. 75°	145	100,000	100,000	100,000	1,600	800	—	Mn, Zn, Cd, Co, Pb, Cu	Fe
9	5-Aminoacridine (for comparison)	m.p. 232°	194	60,000	30,000	7,600	7,600	7,600	960	None	None

— Signifies that growth occurs in M/800 concentration.

* Abstracted from collateral publications (Albert and Magrath, 1947 ; Albert and Gledhill, 1947).

TABLE II.—*Substituted Oxines.*

Highest dilution expressed as $\frac{1}{\text{molarity}}$ of the substance completely preventing visible growth in 48 hours at 37° C.

(Medium : Broth, pH 7·3.)

Number.	Substance.	Criterion of purity.	M.W.	Organisms.						Metals chelated at pH 7·3.*	
				Cl. welchii.	*Str. pyogenes.*	*Staph. aureus.*	*Bact. coli.*	Proteus.	*Ps. pyocyanea.*	Strongly.	Less strongly.
(8)	Oxine (from Table I)	..	..	100,000	100,000	100,000	1,600	800	—	Mn, Zn, Cd, Co, Pb, Cu	Fe
10	8-Methoxyquinoline	m.p. 46° C.	159	—	—	—	—	—	—	None	None
11	2-Methyl-oxine	m.p. 74°	159	13,000	26,000	13,000	800	—	—	Mn, Zn, Cd, Co, Pb, Cu	Fe
12	8-Methoxy-2-methyl-quinoline	m.p. 125°	173	—	—	—	—	—	—	None	None
13	Oxine-N-methochloride	m.p. 170° (d)	196	—	—	—	—	—	—	,,	,,
14	5-Methyl-oxine	m.p. 122°–3°	159	51,000	51,000	51,000	800	—	—	Mn, Zn, Fe, Cd, Co, Pb, Cu	..
15	5-*n*-Propyl-oxine	m.p. 60°–1°	187	100,000	200,000	51,000	—	—	—	Mn, Zn, Fe, Cd, Co, Pb, Cu	..
16	5-Chloro-oxine	m.p. 125°	179·5	100,000	200,000	51,000	6,400	800	—	Mn, Zn, Cd, Co, Pb, Cu	Fe
17	7-Chloro-oxine	m.p. 156°	179·5	200,000	100,000	26,000	1,600	1,600	—	Mn, Zn, Fe, Cd, Co, Pb, Cu	..
18	5 : 7-Dichloro-oxine	m.p. 181°	214	100,000	100,000	26,000	13,000	6,400	1,600	Mn, Zn, Fe, Cd, Co, Pb, Cu	..
19	5-Nitro-oxine	m.p. 178°	190	51,000	100,000	51,000	13,000	3,200	1,600	Fe, Co, Pb, Cu	Zn
20	7-Iodo-5-chloro-oxine (Vioform)	m.p. 178°–9°	305·5	51,000	200,000	13,000	13,000	3,200	—	Fe, Cd, Co, Pb, Cu	Mn, Zn
21	7-Iodo-oxine-5-sulphonic	m.p. 235°(d)	351								

21	7-Iodo-oxine-5-sulphonic acid (Chiniofon, Ferron, Yatren)	m.p. 285°(d)	351	3,200	—	—	—	—	—	None	Chelates hydrophilically but not the oxine type of chelation (see text)
22	Oxine-5-sulphonic acid	m.p. 321°–2°	225	1,600	—	—	—	—	—	,,	(as No. 21)
23	Oxine-5-carboxylic acid	m.p. 273°	189	3,200	—	—	—	—	—	,,	(as No. 21)
24	Ethyl ester of No. 23	m.p. 125°	217	100,000	200,000	13,000	—	—	—	,,	Mn, Zn, Fe, Cd, Co, Pb, Cu
25	4-Amino-oxine	m.p. 208°	160	800	6,400	—	—	—	—	—	Fe, Cu
26	5-Amino-oxine	dihydrochloride analysed for C, H, N, and Cl	160 (base)	6,400	26,000	3,200	—	—	—		
27	7-Amino-oxine	m.p. 124°	160	13,000	13,000	3,200	—	—	—		
28	5 : 7-Diamino-oxine	dihydrochloride analysed for C, H, N, and Cl	175 (base)	6,400	3,200	3,200	800	800	—		
29	5-Diguanidino-oxine	dihydrochloride analysed for C, H, and Cl	244 (base)	—	—	—	—	—	—	Difficult to determine because metal catalyses oxidation of reagent	
30	1 : 2 : 3 : 4-Tetrahydro-oxine	m.p. 121°–2°	149	26,000	1,600	1,600	—	—	—		
31	4-Hydroxy-oxine (8-hydroxy-4-keto-1 : 4-dihydroquinoline)	m.p. 314–5°	161	—	—	—	—	—	—		

— Signifies that growth occurs in M/800 concentration.

* Abstracted from collateral publication (Albert and Magrath, 1947).

bacterial activity. It may be compared with the aminobenzene-sulphonamides and the mono-amino-acridines where one out of three, and two out of five, isomerides respectively are definitely antibacterial.

Some years ago an attempt was made to introduce 3-hydroxyquinoline (No. 3) as a powerful antiseptic on the grounds that it would kill *Staph. aureus* in one second at a dilution of 1 : 4000 (Davin and Schlauch, 1932). However, we find that this dilution will not inhibit growth of the organism in broth.

(2) *Substituted 8-Hydroxyquinolines (Oxines).*

Table II is concerned with the simpler derivatives of oxine. Among these are some in which certain factors which modify chelation (see chemistry section) have been introduced. As already stated in the chemical section of this paper, the chelating power of oxine is totally suppressed when the ionization of the hydroxyl group is prevented or the nitrogen atom made quaternary. Examples of this are shown in Table II, No. 10 and 12, where the ionizable hydroxyl of oxine (No. 8) has been blocked by methylation, and again in No. 13 where the nitrogen of oxine has been made quaternary. It will be seen that in all these cases not only is chelation suppressed, but also antibacterial activity.

The remaining compounds in Table II (No. 14 to 31 inclusive) are variously substituted derivatives of oxine. Although in these substances, the –OH and =N– are free and hence permit of chelation, the extent to which the latter occurs will depend on the deformation exerted by each substituent on the electronic distribution of the molecule. This is discussed in the collateral publication. What is important to note here is that antibacterial action is well maintained in those compounds in which chelation is of the same order as in oxine. However, attention is drawn to the unexpectedly low antibacterial activity of 2-methyl-oxine (No. 11) compared with its isomeride, No. 14, an observation which we consider sheds some light on the mode of attack of these drugs on possible bacterial enzymes and which will be discussed later.

(3) *Compounds Related to Oxine.*

In this section we have classified as best we could a number of substances which display (*a*) the same type of chelation as in oxine, but in a different nucleus, or (*b*) a different type of chelation in the same (i.e. the quinoline) nucleus.

It has already been pointed out that all the structural elements responsible for chelation in the oxine molecule are also present in 1-hydroxyacridine (see Fig. 1). Reference to Table III below shows that 1-hydroxyacridine (No. 32) has strong chelating properties, and, as it is a poorly ionized acridine, chelation apparently determines its antibacterial activity. That this is so is confirmed by the inactivity of 1-methoxyacridine (No. 33), which is also poorly ionized, and in which no –OH is available for chelation. Similar remarks apply to 1-hydroxy-phenazine (No. 34) and its methyl ether (No. 35).

It is interesting to note that 1-hydroxyacridine and 1-hydroxyphenazine do not achieve an antibacterial potency comparable to that of oxine in spite of effective chelation. This is attributed to a steric hindrance to adsorption of these molecules at the metal-bearing surface. The significance of this steric factor, which probably operates against full activity in 2-methyl-oxine (No. 11),

TABLE III.—*Compounds Related to Oxine.*

Highest dilution expressed as $\frac{1}{\text{molarity}}$ of the substance completely preventing visible growth in 48 hours at 37° C.

(Medium : Broth, pH 7·3.)

Number.	Substance.	Criterion of purity.	M.W.	Organisms.						Metals chelated at pH 7·3.*	
				Cl. welchii.	*Str. pyogenes.*	*Staph. aureus.*	*Bact. coli.*	Proteus.	*Ps. pyocyanea.*	Strongly.	Less strongly.
(8)	Oxine (from Table I)	..	..	100,000	100,000	100,000	1,600	800	–	Mn, Zn, Cd, Co, Pb, Cu	Fe
32	1-Hydroxyacridine (Fig. 1)†	m.p. 116° C.	195	16,000	8,000	4,000	–	–	–	Mn, Zn, Cd, Pb, Cu	Fe, Co
33	1-Methoxyacridine	m.p. 134°	209	4,000	–	–	–	–	–	None	None
34	1-Hydroxyphenazine (Fig. 3)	m.p. 157°	196	8,000	4,000	2,000	–	–	–	Mn, Co, Pb	Zn, Cd, Cu
35	1-Methoxyphenazine	m.p. 169°	210	1,000	–	–	–	–	–	None	None
36	2-Hydroxyphenazine	m.p. 253°	196	–	–	–	–	–	–	,,	,,
37	5 : 6-Benzo-oxine	m.p. 106°–7°	195	400,000	200,000	26,000	–	–	–	Mn, Zn, Fe, Cd, Co, Pb, Cu	..
38	6-Hydroxy-*m*-phenanthroline (Fig. 3)	m.p. 159°–60°	196	200,000	100,000	26,000	–	–	–	Mn, Zn, Fe, Cd, Co, Pb, Cu	..
39	8-Mercaptoquinoline (Quinoline-8-thiol)	m.p. 58°–9°	161	200,000	200,000	<3,000	<3,000	<3,000	<3,000	Co, Cu	Mn, Zn, Fe, Cd, Pb
40	8-Aminoquinoline	m.p. 65°	144	–	–	–	–	–	–	None	None
41	Quinoline-2-carboxylic acid (Quinaldinic acid, Fig. 4)	m.p. 157°	173	–	–	–	–	–	–	,,	Zn, Fe, Cd, Pb, Cu
42	Quinoline-4-carboxylic acid (Cinchoninic acid)	m.p. 253°–4°	173	–	–	–	–	–	–	,,	None
43	Quinoline-8-carboxylic acid (Fig. 4)	m.p. 187°	173		–	–	–	–	–	Cu	Pb
44	4-Hydroxy-oxine-2-carboxylic acid (Xanthurenic acid)	m.p. 284°–5°	205	–	–			–	–	(Shows some evidence of hydrophilic chelation, but there is no hydrophobic chelation of the oxine type.)	

– Signifies that growth occurs in M/800 concentration.

* Abstracted from collateral publication (Albert and Magrath, 1947).

† For results showing almost complete lack of antibacterial properties in the four isomerides of this substance see Albert, Rubbo, Goldacre, Davey and Stone (1945).

will be discussed later. It should be noted that benzo- and pyrido-derivatives of oxine have an antibacterial action comparable to their chelate ability (*cf.* 5 : 6-benzo-oxine and 6-hydroxy-*m*-phenanthroline, No. 37 and 38 respectively) but in these examples the extra ring is situated at some distance from the chelating portion of the molecule.

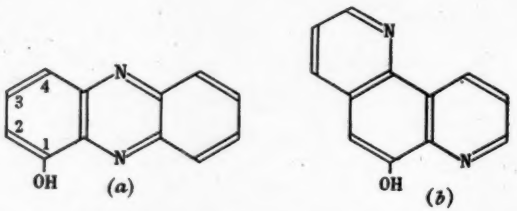

FIG. 3.—(*a*) 1-Hydroxyphenazine. (*b*) 6-Hydroxy-*m*-phenanthroline.

The sulphur analogue of oxine, 8-mercaptoquinoline (No. 39), was difficult to examine because of its poor solubility and ease of oxidation, but it will be seen that its antibacterial activity is of a high order, as would be expected from its chelate properties. Conversely 8-aminoquinoline (No. 40), which does not chelate appreciably, has no antibacterial properties.

Two interesting derivatives of quinoline which form chelate complexes that differ in type from those of oxine will now be discussed. They are quinaldinic acid and quinoline-8-carboxylic acid (No. 41 and 43, Fig. 4 ; *cf.* Fig. 1).

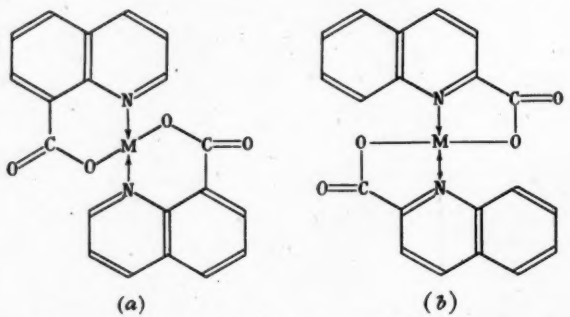

FIG. 4.—Chelate complexes with a divalent metal (M). (*a*) Quinoline-8-carboxylic acid. (*b*) Quinoline-2-carboxylic acid.

These differences in chelate ring structure are reflected in the altered chelating properties (quinoline-8-carboxylic acid (No. 43) being practically a specific agent for copper) and in the absence of any antibacterial activity.

Cinchoninic acid (No. 42), an isomeride of No. 41 and 43, has no chelating properties and is devoid of bacteriostatic qualities. Xanthurenic acid (No. 44), a product of tryptophan metabolism appearing in the urine of rats fed a high protein diet, deficient in pyridoxine, contains the chelate systems of both oxine and quinaldinic acid. It gives colour changes with metals but no precipitates and is not antibacterial.

(4) *Organic Reagents for Metals.*

The circumstantial evidence so far presented supports the hypothesis that the antibacterial activity of oxine and related compounds is dependent on their ability to chelate with metals. In this sense antibacterial action and chemical constitution can be correlated. However, it remains to be shown whether other compounds which chelate with metals, but which differ radically in chemical structure and mechanism of chelation from that of oxine, share the activity of oxine. To this end twenty-seven common organic reagents which chelate with metals were tested in broth using *Bacillus subtilis* and *Bacterium coli* as test organisms. *B. subtilis* was selected as a representative of Gram-positive types, since its surface growth readily overcame the difficulty of detecting growth by turbidity in highly coloured solutions. It will be noted that its sensitivity to oxine is of the same order as other Gram-positive types (Tables I and IV). The results are listed in Table IV below.

It will be noted that none of this representative group, except, perhaps, 1-nitroso-2-naphthol (No. 56), possessed any significant antibacterial activity. The factors involved will be discussed later. It will be sufficient to stress here that chelation is a property common to many substances in the most diverse series, but it cannot, *per se*, induce marked antibacterial activity.

(5) *Effect of pH on Bacteriostasis.*

The importance of pH in modifying acridine action was duly emphasized by Abert *et al.* (1945). It was found that on increasing the hydrogen ion concentration the activity of the highly ionized acridines decreased, and that this decrease was nearly equal to that calculated for a competition between hydrogen ions and drug kations for the same sites on the organisms; that is to say, a change in pH of one unit was balanced by a tenfold change in acridine ion concentration.

In the present series the dissociation constants of members approximately equal to oxine in bacteriostatic activity were determined. It was found that the percentage of anion at pH 7·3 varied between 0·3 per cent (5-*n*-propyl-oxine) and 80 per cent (5 : 7-dichloro-oxine). Two examples, with anionic ionization varying over the pH range of 6 to 9, were examined in buffered broth media in an attempt to detect a competition between oxine anions and hydroxyl ions for the essential metal(s) on the bacterium. The results, listed in Table V, show that under the conditions of these experiments, such competition could not be demonstrated unequivocally. It is possible that another limiting factor, small for oxine and large for 7-chloro-oxine, is concerned.

In the last column of Table V it will be noted that the ratio of hydroxyl ions to drug ions is, in the case of oxine, remarkably constant over a thousandfold variation in hydroxyl ion concentration. This would be consistent with a hypothesis that oxines act on bacteria by competition with hydroxyl ions, but the corresponding ratios for 7-chloro-oxine show enough variation to make this hypothesis distinctly less probable.

(6) *Reversal of Oxine-inhibition by Metals.*

Preliminary experiments recorded below further substantiate the hypothesis that oxine acts by interfering with the metal metabolism of bacteria. As this

TABLE IV.—*Organic Chelate Reagents for Metals.*

Highest dilution expressed as $\frac{1}{\text{molarity}}$ of the substance completely preventing visible growth in 48 hours at 37° C.

(Medium : Broth, pH 7·3.)

Number.	Substance.	M.W.	Organisms.		Metals chelated at pH 7·3.*	
			B. subtilis.	*Bact. coli.*	Strongly.	Less strongly.
(8)	Oxine	145	100,000	1,600	Mn, Zn, Cd, Co, Pb, Cu	Fe
45	Benzoin-oxime (Cupron)	227	—	—	Cu	..
46	Cupferron (Ammonium nitroso-phenyl-hydroxyl-amine)	155	3,200	800	Fe	Cu
47	Dimethylaminobenzal-rhodanine	264	—	—	Cu	Zn, Cd
48	Dimethylglyoxime	116	—	—	None	None†
49	Diphenylcarbazide	242	800	800	Zn, Cu	..
50	Diphenyl-thiocarbazone (Dithizone)	256	3,200	—	Zn, Co, Pb, Cu	Cd
51	Dithiol (3 : 4-Dimercapto-toluene)	156	—	—	Co, Cu	Pb
52	1-Hydroxy-anthraquinone	224	800	800	Cu	..
53	Magneson I (*p*-Nitrobenzene-azo-resorcinol)	259	—	—	None	None†
54	Magneson II (*p*-Nitrobenzene-azo-α-naphthol)	293	—	—	,,	,,†
55	Mercaptobenzthiazole	167	1,600	—	..	Pb, Cu
56	1-Nitroso-2-naphthol	173	6,400	800	Co, Pb, Cu	Mn, Zn, Fe
57	Phenyl-thiohydantoic acid	210	1,600	—	Co	Zn, Cd, Pb
58	Picrolonic acid	264	—	—	None	None†
59	Potassium ethyl-(and sec-butyl-)xanthates	160, 188	1,600	—	Pb, Cu	Cd, Co
60	Rubeanic acid (Dithio-oxamide)	120	800	—	Cu	Zn, Cd, Co, Pb
61	Salicyl-aldoxime	137	—	—	Cu	Fe, Pb
62	Sodium diethyl-dithiocarbamate	171	—	—	Zn, Fe, Cd, Co, Pb, Cu	Mn
63	Sodium dihydroxytartrate osazone	370	—	—	..	Cu
64	Titan yellow	680	—	—	..	Cu
65	Chromotropic acid (Sodium 1 : 8-dihydroxy-naphthalene-3 : 6-disulphonate)	364	—	—	Mn, Pb, Cu	‡
66	α : α′-Dipyridyl	156	3,200	800	Fe	..
67	Nitroso-R-salt (Sodium 1-nitroso-2-naphthol-3 : 6-disulphonate)	377	1,600	—	Fe, Co, Cu	..
68	*o*-Phenanthroline	180	12,800	800	Fe	..
69	Quinalizarin (Tetrahydroxyanthraquinone)	272	—	—	Mn, Fe, Co, Pb	Zn, Cd, Cu
70	Thioglycollic acid	92	—	—	Co	..

— Signifies that growth occurs in M/800 concentration.

* Abstracted from collateral publication (Albert and Gledhill, 1947).

† Under other (non-physiological) conditions, these substances are important chelate reagents for traces of metals.

‡ In Nos. 65–70 (inc.) the metal chelate-complex is water-soluble.

TABLE V.—*The Effect of pH on Ionization and on Antibacterial Activity.*

Highest dilutions completely preventing visible growth in 48 hours at 37° C.

(Organism : *Staphylococcus aureus*. Medium : Buffered broth.)

Substance.	Buffer M/40.	pH.	Hydroxyl ion concentration. A.	Percentage of drug present as anion. B.	Concentration of drug necessary for bacteriostasis. C.	Concentration of drug anion necessary for bacteriostasis. D.	A/D.
Oxine (pK = 9·45)	Phosphate	6	N/100,000,000	0·04	M/100,000	M/250,000,000	2·5
	,,	7	10,000,000	0·5	100,000	20,000,000	2·0
	,,	8	1,000,000	3·5	100,000	2,900,000	2·9
	Borate	8	1,000,000	3·5	100,000	2,900,000	2·9
	,,	9	100,000	26·0	100,000	380,000	3·8
7-Chloro-oxine (pK = 7·7)	Phosphate	6	N/100,000,000	2·2	M/100,000	M/4,500,000	0·045
	,,	7	10,000,000	16·6	100,000*	600,000	0·06
	,,	8	1,000,000	66·6	100,000	150,000	0·15
	Borate	8	1,000,000	66·6	100,000	150,000	0·15
	,,	9	100,000	95·0	200,000	210,000	2·10

A : Calculated from pH determined by glass electrodes.
B : Calculated from pH and pK_a.
C : Experimentally determined.
D : Calculated from B and C.

* 7-Chloro-oxine was repeatedly found not to inhibit this organism below M/26,000 when tested in naturally buffered broth at pH 7·3 (Table II). The fourfold increase in activity in M/40 phosphate buffer (see above) was repeatedly observed and may be connected with the high saline content of the medium.

aspect of our work is to be more fully described in a later publication, it will suffice to say that the system for demonstrating reversal was set up as follows: To a series of inoculated media, the bacteriostatic concentrations of oxine for each species under test were added, followed immediately by the addition of four equivalents of metal in the form of appropriate salts. The tubes were then incubated for 24 and 48 hours and the presence of growth noted by turbidity. Table VI below sets out the findings for a typical experiment.

TABLE VI.—*The Effect of Metals in Reversing the Inhibitory Action of Oxine.*

Medium: Broth.

Organism.	Medium contains—		Divalent Metals as Salts.								
	Oxine 1/M.	Metal 1/M.	Ca.	Mg.	Mn.	Zn.	Fe.	Cd.	Co.	Cu.	Absent.
Strep. pyogenes	100,000	50,000	—	—	—	—	—	0	++	—	—
Staph. aureus	100,000	50,000	—	—	—	—	—	—	++	—	—
Bact. coli	1,600	800	—	—	—	++	++	—	—	++	—
Proteus sp.	400	200	—	—	—	++	++	—	—	—	—

0 signifies metal alone too toxic to test.
— „ no visible growth.
++ „ moderate growth.

It will be noted that a sharp difference between Gram-positive and Gram-negative types is revealed in that only cobalt is effective in promoting growth of Gram-positive bacteria in the presence of inhibitory concentrations of oxine, whereas zinc and iron reverse the action of oxine for the Gram-negative species. The significance of this distinction between two important classes of micro-organisms is at present under consideration.

(7) *Comparison of the Sensitivity of Gram-positive and Gram-negative Types to Oxine.*

In view of the marked sensitivity of the Gram-positive bacteria so far examined and their specific protection by cobalt, it seemed worth while to extend our investigation of oxine inhibition to a wide range of bacterial types. To this end twenty species were examined representing ten Gram-positive genera and ten Gram-negative genera. The results are listed in Table VII.

It will be noted that in general Gram-negative bacilli, except *Haemophilus pertussis* and the Gram-negative coccus, are fairly resistant to oxine, whereas the Gram-positive types are sensitive to its action. This separation of microbial types is reminiscent of a similar selectivity of penicillin activity, except that important differences are to be found in the genus *Haemophilus*. This evidence alone suggests that oxine, in spite of its penicillin-like spectrum, is acting in a manner distinct from that of penicillin. Further, comparison of this table with a practically identical one for the acridine series (Albert, Rubbo, Goldacre, Davey and Stone, 1945) shows that oxine activity is quite distinct from that of the acridines.

(8) *The Effect of Serum Protein on Antibacterial Activity.*

It is possible that some of the compounds described here will stimulate interest in respect to their clinical potentialities, particularly in view of the very marked activity of the key substance, oxine, of this series (Table VII). In order to bring these findings in line with our previous work (Parts I and II), it was decided to re-test some of the significant compounds in 10 per cent serum broth. A

TABLE VII.—*The Sensitivity of Gram-positive and Gram-negative Bacteria Towards Oxine.*

Highest dilutions completely preventing visible growth in 48 hours at 37° C.
(Medium : Broth, pH 7·3.)

Gram-positive species.	Inhibitory dilutions.		Gram-negative species.	Inhibitory dilutions.	
	1/molarity.	W/V dilution.		1/molarity.	W/V dilution.
Cl. welchii	100,000	680,000	*Bact. coli*	1,600	10,000
Cl. sporogenes	100,000	680,000	Proteus sp.	800	5,000
B. subtilis	50,000	340,000	*Ps. pyocyanea*	< 800	< 5,000
Str. pneumoniae	100,000	680,000	*Salm. typhi*	3,000	20,000
Str. pyogenes	100,000	680,000	*Bact. flexneri*	13,000	90,000
Str. viridans	200,000	1,360,000	*V. cholerae*	2,000	13,000
Staph. aureus	100,000	680,000	Pasteurella	6,000	40,000
Lactobacillus	50,000	340,000	*Br. abortus*	26,000	180,000
C. diphtheriae	200,000	1,360,000	*H. pertussis*	200,000	1,360,000
Myco. phlei	26,000	180,000	*N. meningitidis*	200,000	1,360,000

comparison of the activity of these derivatives in presence and absence of serum is indicated in Table VIII.

It will be seen that the influence of serum in quenching antibacterial activity is negligible in the case of several oxine derivatives (No. 8, 14 and 19), slight for No. 15 and 18, and in the cases of 5 : 6-benzo-oxine and 6-hydroxy-*m*-phenanthroline the activity is significantly reduced. This loss of activity is due no doubt to adsorption at the serum-protein/water interface, and would seriously prejudice the success of these substances as chemotherapeutic agents.

DISCUSSION.

Our present endeavour has been to focus attention on the chemical property of chelation, and to relate this to the degree of antibacterial activity shown by oxine and related compounds. In brief, it has been shown that of the seven isomeric mono-hydroxyquinolines, only oxine, the 8-isomeride, can chelate, and it alone is bacteriostatic (Table I).

The chelating power of these, and all other compounds in the Tables, has been determined by a semi-quantitative method involving the reaction, in turn, of the following metallic ions as described in the collateral publications : Calcium, magnesium, manganese, zinc, iron, cadmium, cobalt, lead and copper.

The results, both for the above series of isomerides and for the derivatives of oxine, show a remarkably positive correlation between the ability of a substance of the oxine type to chelate with metals and the degree of antibacterial activity which it possesses. Hata's suggestion (1932) that oxine owes its antibacterial properties to a combination of those of quinoline and phenol in the one molecule can no longer be seriously entertained in so far as neither quinoline (No. 1) nor phenol is antibacterial at a dilution of 1 : 5000, and the isomerides of oxine (No. 2–7 inclusive), although phenols, are not antibacterial.

Structural modifications of oxine which adversely modify antibacterial action would seem to do so by various mechanisms, of which the following appear to be important :

(*a*) Affecting chelation adversely (e.g. No. 2–7, 10 and 13).

(*b*) Altering type of chelation from hydrophobic to hydrophilic (e.g. 22 and 23).

(*c*) Introducing steric hindrance to chelation *at a surface* (e.g. 11 and 32).

TABLE VIII.—*The Effect of Serum Protein on Antibacterial Activity.*

Highest dilution expressed as $\frac{1}{\text{molarity}}$ of the substance completely preventing visible growth in 48 hours at 37° C.

Number.	Substance.	Medium.	Organisms.					Depression of activity by serum protein.
			Cl. welchii.	*Str. pyogenes.*	*Staph. aureus.*	*Bact. coli.*	Proteus.	
8	Oxine	Broth	100,000	100,000	100,000	1,600	800	None
		Serum broth	100,000	100,000	100,000	1,600	800	
14	5-Methyl-oxine	Broth	51,000	51,000	51,000	800	—	,,
		Serum broth	51,000	51,000	51,000	—	—	
19	5-Nitro-oxine	Broth	51,000	100,000	51,000	13,000	3,200	,,
		Serum broth	51,000	100,000	51,000	6,400	6,400	
15	5-*n*-Propyl-oxine	Broth	100,000	200,000	51,000	—	—	Slight
		Serum broth	51,000	26,000	26,000	—	—	
18	5 : 7-Dichloro-oxine	Broth	100,000	100,000	26,000	13,000	6,400	,,
		Serum broth	51,000	51,000	26,000	6,400	3,200	
37	5 : 6-Benzo-oxine	Broth	400,000	200,000	26,000	—	—	Marked
		Serum broth	3,200	1,600	800	—	—	
38	6-Hydroxy-*m*-phenanthroline	Broth	200,000	100,000	26,000	—	—	,,
		Serum broth	6,400	6,400	6,400	—	—	

— Signifies that growth occurs in M/800 concentration.

As regards (*b*), although the introduction of a sulphonic or carboxylic acid group into the oxine nucleus (as in No. 22 and 23) prevents the formation of water-insoluble complexes and almost abolishes antibacterial activity, the latter can be restored to a figure comparable to that of oxine when the " hydrophobic " type of chelation is restored, e.g. by esterification (*cf.* ethyl ester of oxine-5-carboxylic acid, No. 24).

As regards (*c*), the adverse effect of nuclear substitution by inert groups not affecting chelation (e.g. an alkyl group or benzene ring) is readily seen in 2-methyl-oxine (No. 11) and 1-hydroxyacridine (No. 32 ; *cf.* Fig. 1). However, similar inert groups occupying positions which are spatially separated from the chelate groups (*cf.* 5-methyl-oxine, 5 : 6-benzo-oxine and 6-hydroxy-*m*-phenanthroline ; No. 14, 37 and 38 respectively) do not exert this adverse effect.

A similar steric inhibition of activity has been noted by Krichevski, Sternberg and Galperin (1935), who found inactivation of certain quinoline antimalarials was caused by the insertion of a methyl group in the 2-position.

The poor antibacterial action of many organic reagents for metals (Table IV), particularly those substances which are not inferior to oxine in chelating power, further emphasizes the importance of other factors, especially steric factors, in determining activity.

This raises the question as to whether oxine inhibits bacterial growth by chelating with metals in the medium or on the bacterial surface. The balance of evidence strongly favours the latter alternative. For example, it has been amply shown here that substances with the same chelate properties may have quite different degrees of antibacterial activity. Again, removal of heavy metal ions from the broth medium by repeated treatment with oxine and subsequent removal of the latter with chloroform, as in the technique of Waring and Werkman (1942), does not impair the growth of test organisms, nor are the antibacterial titres for oxine diminished in the treated medium. These results (to be reported on later) indicate that the organism growing in nutrient broth may derive its requirements of heavy metals by metabolizing the natural chelate complexes (almost certainly formed from amino acids and peptides) in the medium. It is, therefore, suggested that oxine exerts its antibacterial action by combining with,

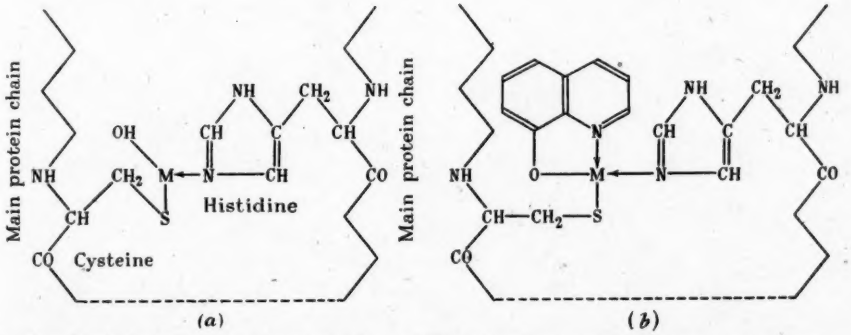

FIG. 5.—Conjectural location of a divalent metal (M) in enzyme attacked by oxine, (*a*) before and (*b*) after attack. Glutamic acid may replace cysteine and arginine may replace histidine, and so on.

and hence inactivating, an ionized metal forming part of a possible cellular enzyme. Fig. 5, which is speculative, indicates how the metal, and hence the oxine, may be attached to the cell.

Regarding the identity of the metal, M, indicated in Fig. 5, we have insufficient evidence to reach a decision yet. Preliminary experiments, reported here (Table VI), show that only cobalt reverses the action of oxine for two Gram-positive species, *Staphylococcus aureus* and *Streptococcus pyogenes*, whereas in the case of *Bacterium coli* and *Proteus*, zinc, iron and copper, and zinc and iron respectively reverse the action of oxine for these Gram-negative species. The specific reversal by cobalt for these (and other) Gram-positive organisms raises many interesting points which will be discussed in a later publication.

CLINICAL APPLICATION OF THE PRESENT STUDIES.

Oxine has been used clinically for fifty years as a surface antibacterial and fungicide, and many modifications of the drug have been tried (Altpeter, 1929). Nevertheless, it is a drug which is seldom prescribed to-day. The greatest disadvantage attending its topical application is the strong acidity of the solutions commonly dispensed (pH 3).

This acidity is due to the traditional prescribing of oxine as its acidic sulphate, i.e. as chinosol or the so-called potassium hydroxyquinoline sulphate of the B.P.C. It is preferable to order pure oxine, which forms neutral solutions (e.g. 1 : 2000 in water or 1 : 1000 in 15 per cent. alcohol).

Now that the action of this drug has been correlated with chelation and the effects of chemical substitution have been explored, the way is made clear to reinvestigate the clinical potentialities of derivatives of the parent molecule.

In effect, this investigation is pharmacological rather than bacteriological. The toxicities of some members of the present series, determined by one of us (S.D.R.), indicate that the 5-methyl derivative of oxine (No. 14) offers greatest hope for clinical application because of its surprisingly low toxicity (L.D. 50 is *ca.* 0·60 g. per kg. (s/c mouse) compared with 0·09 g. per kg. for oxine). The clinical application of this compound is at present under investigation. For example, the great sensitivity of *Haemophilus pertussis* to oxine suggests that less toxic members of similar activity, viz. 5-methyl-oxine, might be of value in the treatment of pertussis infections. Furthermore, the tuberculo-chemotherapeutic investigation of the oxines has been commenced because of encouraging results already obtained *in vitro*. These aspects will be reported on separately.

The iodine-containing derivatives (No. 20 and 21, Table II) are of interest because of their very successful employment in treating amoebic dysentery. It is gathered from the literature that there is not much to choose between these drugs in amoebiasis; but a very clear-cut difference between them is seen in that whereas vioform (No. 20) has chelating and antibacterial properties qualitatively and quantitatively comparable to those of oxine, chiniofon (No. 21) does not chelate in this way and is not antibacterial. It is quite evident then that the chelation which is essential for the antibacterial action of oxine drugs is not the source of their efficacy as amoebicides. The feature which these two drugs have in common is that they split off iodine with surprising readiness, instantly when boiled with water, and more slowly when the solutions are kept at room temperature. Since it is known that the corresponding chlorine analogues are not effective amoebicides (von Oettingen, 1933), it seems likely that these types of drugs owe their activity to the weakness of the carbon-iodine bond in the 7-

position of the oxine nucleus, and to the steady evolution of inorganic iodine that ensues. This is true also of diodoquin (5 : 7-di-iodo-oxine), which was found too insoluble for inclusion in the present study.

Hence the derivatives of oxine provide one more striking example of how two groups of drugs, which from the point of view of structural chemistry appear to contain the same features, actually are found to depend on quite distinct properties for their respective activities (*cf.* marfanil and sulphanilamide; 1-hydroxyacridine and 5-aminoacridine).

SUMMARY.

1. A bacteriological examination has been made of forty-three compounds related to oxine (8-hydroxyquinoline), and of twenty-four compounds forming a heterogeneous selection of organic reagents for metal ions.

2. A strong positive correlation between antibacterial activity and chelating powers has been demonstrated in the oxine series (Tables I and II). That is to say, the activity of this type of drug is determined by its ability to combine with polyvalent metals and form complexes lacking the characteristic properties of metal ions.

3. It is suggested that the active members of this series function by disorganizing the trace-metal mechanism essential for bacterial growth, and that this effect depends on combination between the drug and some metal(s) on the bacterial surface.

4. Whereas the ability to chelate is shared by many organic reagents, the ability to function as an antibacterial substance is largely restricted to oxine and some related compounds (Tables III and IV). Thus antibacterial activity among chelating compounds is determined not only by the ability to chelate, but also by the molecular architecture.

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THE *IN VIVO* ACTIVITY OF PENICILLIN ESTERS.

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AT a comparatively early stage in the development of penicillin it was realized that its esters might, by virtue of their greater stability, offer advantages over its salts, the form in which it had been almost exclusively used for therapeutic purposes. It was further suggested by Mayer, Hobby and Dawson (1943) that oil suspensions of the esters would give slower absorption and excretion than aqueous solution of the salts, and would thus maintain for a longer time the requisite concentration in the plasma after intramuscular injection.

The methyl, ethyl, *n*-butyl and benzhydryl esters of penicillin were first prepared by Mayer, Hobby and Chaffee (1943), who reported that they were active in infected mice, and showed that they were hydrolyzed in the animals' bodies with liberation of free penicillin. They stated further that the esters were not destroyed in the stomach, and that oral administration was therefore effective.

Early attempts by us to repeat this and other published work gave inconsistent results, probably owing to the marked and varying impurity of the esters then available to us. Subsequently we received from the Chemical Research Department of this Company supplies of pure methyl and benzyl esters, which were stable for many months. The experimental results here recorded were carried out with this material.

EXPERIMENTAL.

Methods and Materials.

Estimation of penicillin.

The penicillin content of blood samples was estimated by the capillary tube method of Fleming (1944), using *Streptococcus haemolyticus* as test organism. Penicillin in other materials and extracts was determined by the dilution method of Abraham, Chain, Fletcher, Gardner, Heatley, Jennings and Florey (1941): the test organism was *Staphylococcus aureus* (Oxford strain, N.C.T.C. No. 6571A) on a papain digest broth medium. The results are expressed throughout in terms of penicillin International Units (I.U.).

Penicillin derivatives.

Esters.—The penicillin methyl ester is a white crystalline compound, insoluble in water but soluble in arachis oil and fat solvents, having a potency of 1500 I.U./mg. In this paper the doses of ester used are given for convenience in penicillin units, although the esters are themselves biologically inactive. The number of units assigned to a given dose of ester is the number derived from

that dose after theoretically quantitative hydrolysis into penicillin. In this sense the methyl ester has an "activity" of 1490 I.U./mg. and the benzyl ester 1390 I.U./mg. The arachis oil solution was best prepared by prolonged grinding of the ester with the oil until the mixture appeared homogeneous.

The penicillin benzyl ester is a yellow oil, with solubilities similar to those of the methyl ester. The solution in oil was best prepared by dissolving in ether, adding the necessary volume of arachis oil and removing the ether under reduced pressure.

Both substances were tested *in vitro* by the dilution method; both were found completely inactive. It seems clear from this that neither substance is hydrolyzed in water.

Sodium salt.—The penicillin sodium salt used was a commercial specimen of potency *ca.* 1200 I.U./mg. (pure penicillin contains 1660 I.U./mg.).

Protection of Mice Infected with Streptococcus haemolyticus.

The relative protective effects of sodium penicillin and penicillin methyl ester were examined by comparing their actions in mice infected intraperitoneally with fifty lethal doses of a haemolytic streptococcus (Strain 618). Each substance was prepared as an arachis oil solution or homogeneous suspension containing 4000 I.U./ml. The results of administering 0·2 ml. of these preparations (800 I.U.) intramuscularly three times daily to mice (in groups of six) are given in Table I. It would appear that, with these doses, both substances afforded complete protection.

TABLE I.—*Protective Effect of Penicillin Derivatives against Streptococci in Mice.*

Treatment was stopped after 3 days.

Penicillin derivative.	Infected.	Number of mice in group.	Number of mice surviving. Days after infection.					
			1.	2.	3.	4.	5.	6.
Methyl ester	No	3	3	3	3	3	3	3
,, ,,	Yes	6	6	6	6	6	6	5
Sodium salt	,,	6	6	6	6	6	6	6
None	,,	3	1	0	0	0	0	0

In another experiment designed to detect any difference that resulted from altering the dose, preparations containing 8000 I.U./ml. were administered in quantities of 0·1 ml. Infected mice were treated once, twice or three times daily. The uninfected animals served as a toxicity control, and received ester or salt three times daily. Otherwise, conditions were identical with those of the previous experiment. Results are given in Table II.

It is clear that the protective effects of sodium penicillin and penicillin methyl ester (both given in oil) are identical at these doses under the conditions of the experiment.

We next turned our attention to the rabbit, because this species is used in most of our biological examinations. Four animals in a group each received intramuscular injections of 40,000 I.U. either of an ester in oil solution or of unesterified penicillin (as control). Blood and urine samples were collected

TABLE II.—*Effect of Varying the Dose of Penicillin Derivatives.*

Treatment was stopped after 3 days. Each dose contained 800 I.U. penicillin.

Penicillin derivative.	Number of doses per day.	Infected.	Number of mice in group.	Number of mice surviving. Days after infection. 1.	2.	3.	4.	5.	6.
Methyl ester	3	No	3	3	3	3	3	3	3
	3	Yes	6	6	6	6	5	5	5
	2	,,	6	6	6	6	5	5	5
	1	,,	6	6	6	6	6	6	6
Sodium salt	2	No	3	3	3	3	3	3	3
	3	Yes	6	6	6	6	6	6	6
	2	,,	6	6	6	6	6	6	6
	1	,,	6	6	5	5	5	5	5
None	—	,,	3	1	0	0	0	0	0

from every animal at 0·5, 1, 2, 3·5, 5, 7·5 and 24 hr. after injection and the penicillin content was estimated. Water was given by mouth during the experiment to ensure adequate diuresis. It was found that neither with the methyl nor with the benzyl ester was a significant level of penicillin present in the blood at any time during the 24 hr. following injection. Occasionally a small amount (0·08 I.U./ml.) was found. Negligible amounts were excreted in the urine.

It appeared that the injection of penicillin esters into the rabbit did not lead to the circulation of free penicillin in the blood or its excretion in the urine. It is possible that the arachis oil was preventing absorption of the penicillin methyl ester: a group of three rabbits were therefore given an intramuscular injection of a suspension made by prolonged grinding of 10 mg. ester with 2 ml. physiological saline. Again no free penicillin was detected in the blood or urine over a period of 24 hr.

The apparent species difference found between mice and rabbits led us to investigate the behaviour of the ester in two other species of animals (rats and guinea-pigs). These were examined along with groups of mice and rabbits; the results are summarized in Table III. In all instances injection was by the intramuscular route and blood was collected by heart puncture. This experi-

TABLE III.—*Free Penicillin in Blood of Various Animals after Injection of Penicillin Methyl Ester.*

The ester used had a potency of 1500 I.U./mg.

Animal.	Amount ester injected (units).	Penicillin in blood (I.U./ml.). Hours after injection. 1.	2¼.	4.	7.	24.	36.	48.
Rabbit	15,000	0	0	0	0	0	0	0
Guinea-pig	2,500	1·0	0·3	0·25	0	0	0	—
Rat	1,500	0·3	0·06	0·25	0	0	0	—
Mouse	300	0·25	0·25	0·06	0	0	—	—

ment shows clearly that penicillin methyl ester is split in the bodies of guinea-pigs, rats and mice, but not of rabbits. Repetition of this experiment on two further occasions, under identical conditions, gave similar results: free penicillin was present in the blood of all the animals, except rabbits, up to 7 hr. after injection and in the urine up to 24 hr.

Hydrolysis of Penicillin Esters.

It seemed possible that the above results were to be explained by the existence in guinea-pigs, rats and mice of an esterase capable of hydrolyzing penicillin. Apart from its biological interest, such an esterase would be of use in the assay of penicillin esters: the hydrolysis of these substances by water is very slow, and more drastic hydrolytic agents cannot be used without destruction of the liberated penicillin.

Crude extracts were made by mixing with distilled water the macerated liver or kidneys of rats. After standing in the refrigerator overnight the macerate was filtered and the filtrate sterilized by Seitz filtration. The filtrates had little or no effect *in vitro* on penicillin methyl ester.

It seemed logical next to examine the blood of guinea-pigs, rats and mice. Rabbit and human bloods were also examined for comparison, coagulation being prevented by the addition of heparin (1 mg./l.). At this concentration heparin was found to have no effect on the ester. Preliminary investigations, as recorded in Table IV, showed clearly that human and rabbit blood had no effect on the penicillin methyl ester, but that hydrolysis was almost complete 30 min. after the addition of blood from guinea-pig, rat or mouse, rat blood being slightly more active than the others.

It seemed likely, therefore, that the presence of an esterase in the blood of guinea-pig, rat and mouse accounted for the protective effect of penicillin esters

TABLE IV.—*Hydrolysis of the Methyl Ester of Penicillin by Blood of Various Animals.*

The diluted ester was added to the blood inoculated with the test organism (as in the capillary tube assay method for penicillin in blood). The presence of esterase is indicated by the inhibition of growth by the penicillin released.

++ and + = growth. 0 = no growth.

Penicillin derivative.	Source of blood.	Final dilution of ester (or Na penicillin) solution in blood.						
		1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.
Methyl ester (2 I.U./ml.)	Rat	0	0	0	0	0	+	++
	Guinea-pig	0	0	0	0	+	++	++
	Mouse	0	0	0	0	+	++	++
	Rabbit	++	++	++	++	++	++	++
	Man	++	++	++	++	++	++	++
	None (control)	++	++	++	++	++	++	++
Sodium salt (2 I.U./ml.)	None (control)	0	0	0	0	0	+	++

in these species, but that neither rabbit nor man has such an enzyme, with the result that penicillin esters are ineffective on them.

It was found that the activity of the plasma esterase was destroyed by heating the plasma (or serum) to 70° C. for 30 min., and also by the action of iodoacetic acid, of which a concentration of 0·1 g./l. in the plasma reduced the activity of the esterase in 1 hr. to one-fifth of the original value.

Behaviour of methyl ester in human subjects.

Each of four volunteers received intramuscularly 0·8 ml. arachis oil suspension, containing 100,000 I.U. penicillin methyl ester. Blood samples were taken at intervals during the following 24 hr., and urine was collected during the same period, the volumes being recorded. It was found that there was no penicillin circulating in the blood of any of the subjects at various intervals between 1 and 24 hr. after injection. Only about 1000 I.U. penicillin were found altogether in the urine of each. These results appeared to confirm the conclusion that penicillin methyl ester was hydrolyzed to a negligible extent, if at all, in the human body.

Stability of methyl ester in vivo.

It remained to be determined whether or not penicillin methyl ester is absorbed, circulates in the blood stream and is excreted unchanged in the urine. In that event, it would escape detection by the ordinary procedures, the ester being inactive *in vitro* against the usual test organisms. Two rabbits were therefore given intramuscular injections of 20,000 I.U. penicillin methyl ester in 0·5 ml. arachis oil. Samples of blood and urine were collected at intervals up to 24 hr. Each sample was then titrated twice, (*a*) by the usual procedure for the presence of free penicillin, and (*b*) for the presence of penicillin after incubation with guinea-pig plasma diluted one in ten. The esterase activity of the plasma was established by noting its action at the same concentration on a known amount of the methyl ester. No penicillin was found either in blood or urine, either before or after incubation with guinea-pig plasma. It would seem that unchanged methyl ester does not circulate unhydrolyzed in the rabbit after intramuscular injection.

Penicillin benzyl ester.

Experiments on the hydrolysis of this ester by rat, guinea-pig, rabbit and mouse plasma gave results closely similar to those obtained with the methyl ester. A suspension of the benzyl ester containing 2·0 I.U./ml. was completely hydrolyzed in 1 hr. by rat plasma, and about 50 per cent by guinea-pig plasma in the same time. Neither human nor rabbit plasma had any effect. Experiments with two groups of rabbits showed that penicillin benzyl ester in oil suspension gave, after intramuscular injection, no free penicillin in blood or urine. It seems likely that these conditions would also apply to man.

Oral Administration of Penicillin Methyl Ester.

Four rabbits were given 2 ml. arachis oil solution of penicillin methyl ester (containing 40,000 I.U.) by passing it through a rubber catheter into the stomach ; a further 30 ml. water were also given by catheter after 4 hr. The usual collection of blood and urine specimens was made at regular intervals, and a representative

specimen of faeces was also collected after 7 and 24 hr. Samples (1 g.) of the faeces were extracted with 20 ml. broth at room temperature for 4 hr. by emulsification in a mechanical shaker. After centrifuging, the supernatant was divided into two parts: the first was sterilized by Seitz filtration and titrated for penicillin content; the other was titrated in the same way after treatment for 1 hr. at room temperature with 1 ml. guinea-pig serum/20 ml. In one of the rabbits traces of penicillin were found in the blood during the first hour after administration, but no penicillin was found in any other sample of blood or urine. Three control rabbits that had received the same quantity of sodium penicillin in arachis oil had an effective blood level for the first hour only, but excreted penicillin in the urine during the first four hours. The samples of faeces contained less than 1 I.U./g. free penicillin either before or after incubation with esterase, indicating that the methyl ester does not pass unchanged through the intestines.

Two human volunteers each ingested in the morning, after breakfast, 100 mg. arachis oil suspension containing 150,000 I.U. penicillin methyl ester. Blood and urine samples were taken at regular intervals over 24 hr. No penicillin activity was found in any of them.

SUMMARY.

(1) There is a species difference in the extent to which the methyl or the benzyl ester of penicillin protects animals against penicillin-sensitive organisms.

(2) Mice, rats and guinea-pigs have a penicillin esterase in the blood serum; no such esterase is present in the sera of man and rabbit.

(3) The serum esterase can be used to estimate the penicillin esters; the penicillin liberated on hydrolysis is assayed by the usual methods.

(4) It is concluded that penicillin esters are unlikely to have therapeutic value in man by systemic or oral route.

I wish to acknowledge the co-operation of Mr. J. C. Clayton, who prepared the penicillin esters in the Chemical Research Laboratory of Glaxo Laboratories, Ltd., and that of Mr. P. Muggleton, who assisted me throughout the work.

Note.

While this paper was being prepared, we saw the publication of Richardson, Walker, Miller and Hansen (1945). These authors tested penicillin methyl and benzyl esters and arrived at conclusions similar to our own. The note by Cavillito, Krichner, Miller, Bailey, Elinek, Warner, Suter and Tainter (1945) reached us while our work was in progress. It is concerned only with the activity of the benzyl ester in mice. Our results confirm theirs.

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THE PENICILLIN PRODUCED BY *ASPERGILLUS PARASITICUS*.

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IN a preliminary communication (Cook and Lacey, 1944) the production of an antibiotic resembling penicillin, and provisionally termed "parasiticin," was described. This was a metabolic product of *Aspergillus parasiticus* when grown under selected conditions, different conditions giving rise to other antibiotics (Cook and Lacey, 1945). The present paper records some properties of "parasiticin," its production on a larger scale, and experiments leading to the final identification of the major constituent of the material as penicillin-II (penicillin-G or benzylpenicillin).

EXPERIMENTAL.

General Outline of the Work.

For this work surface cultures of *A. parasiticus* were best grown on a medium containing corn-steep liquor. Several other media which were tried later containing phenylacetic acid and derivatives of it produced no activity, although it is known that at least some strains of *Penicillium* sp. can be induced to biosynthesize penicillin-II in this way (Higuchi, Jarvis, Peterson and Johnson, 1946). Under optimum conditions of strain, medium and incubation the culture solution inhibited the growth of *Staph. aureus* at a limiting dilution of 1 : 600 and contained about 15 Oxford units/c.c.* of "penicillin," though an average yield was 7·5–10 units/c.c. Batches of about 10 l. were given preliminary purification by adsorption of the active material on charcoal, elution, extraction from acidified aqueous solution by chloroform, and recovery as a barium or sodium salt with a potency of *ca.* 300 units/mg. During these and subsequent operations the material behaved entirely like crude penicillin, giving rise on occasion to troublesome and sometimes unaccountable losses, and all the customary precautions had to be observed. It was further purified by partition chromatography on columns of silica-gel.

Owing to the difficulty of producing large quantities of the antibiotic under laboratory conditions the maximum potency attained was never greater than 500 units/mg. (i.e. *ca.* 30 per cent active penicillin). The material could not be crystallized under conditions which suffice for what is now known to be the same penicillin of similar potency from *P. notatum*, a difference to be ascribed to the differing nature of the impurities.

The penicillin nature of the antibiotic was apparent from its complete inactivation by penicillinase. Its identification followed from both its biological and chemical properties. Thus for differential assay, solutions each containing 40 units/c.c. (with reference to *Staph. aureus*) were made of "parasiticin" and

* Penicillin values are given as "Oxford" units throughout.

authentic penicillin-II ; they both permitted a trace of growth of *Staph. aureus* at a dilution of 1 : 2000 and full growth at 1 : 4000. The solutions were active to similar degrees among themselves towards *B. subtilis* (1 : 80), *E. coli* (1 : 5) and *Streptococcus pyogenes* (1 : 10).

It was known that at least certain preparations of impure penicillin were less readily crystallized than converted (by standing at pH 2) into crystalline but biologically inactive " penillic acid " (Duffin and Smith, 1943), and solutions of " parasiticin " were therefore treated in this way. Formation of a penillic acid was indicated by the appearance of characteristic ultra-violet light absorption, and a crystalline acid was eventually isolated in pure condition together with less pure and apparently mixed penillic acids.

The main " parasiticin " penillic acid had a crystal appearance resembling penillic-II acid but differing from penillic-I, dihydropenillic-I and penillic-III acids. Although depressions in melting point on mixing penillic acids are not large, only a mixture of the present material with penillic-II acid showed no depression in melting point ; moreover " parasiticin " penillic acid gave analytical figures to be expected of penillic-II acid (Anon., 1945). The X-ray diffraction diagram was kindly examined for us by Dr. D. Crowfoot and Dr. B. W. Rogers (Laboratory of Chemical Crystallography, University of Oxford), who reported that it gave single crystal and powder photographs characteristic of penillic-II acid. It was thought that one preparation may have been contaminated with penillic-III acid.

Production of the Antibiotic.

The selected strain of *A. parasiticus* was subcultured on malt-agar, and the freely sporing growth after 10 days at 20–25° C. used to inoculate the liquid media.

The following medium was adopted for large-scale cultures : $NaNO_3$, 20 g. ; KCl, 5 g. ; $MgSO_4$, 5 g. ; K_2HPO_4, 10 g. ; $FeSO_4$, $7H_2O$, 0·1 g. ; corn-steep liquor, 750 c.c. ; glucose, 200 g. ; distilled water to 10 l.

The medium was brought to pH 7 with Na_2CO_3(anhydrous) (*ca.* 40 g.), distributed into quart-size milk bottles in 400 c.c. portions, sterilized at 15 lb. pressure for 15 min., inoculated and incubated at 20–25° C. Activity towards *Staph. aureus* appeared after 6–7 days, was maximum at *ca.* 14–16 days, and almost disappeared after a further 3 days. The maximum antibacterial titre observed was 1 : 600, though often this was not attained.

Concentration and Purification.

A typical preliminary concentration was carried out in the following way: The fermented medium (10 l., 10 units/c.c.) was filtered in four portions through 6-in. filter beds composed of charcoal (35 g.) and kieselguhr (35 g.). Without allowing the filter beds to dry they were eluted with a total of 1 l. 60 per cent acetone and 1 l. ethyl acetate saturated with water (recovery, 50,000 units or 50 per cent). The eluates were stirred at 0° C. with chloroform (850 c.c.) ; the aqueous layer was brought to pH 2 by adding 10 per cent v/v H_3PO_4 dropwise, saturated with NaCl and the chloroform solution containing all the activity run off. The chloroform solution contained 42,500 units (85 per cent), and was stirred with water and 0·33 N $Ba(OH)_2$ until the solution was neutralized, thus giving a crude barium salt (300 units/mg.) in aqueous solution. In earlier work

this barium salt (187,500 units) was converted into the penillic acid by the method described below, but the product was not crystalline. In other experiments a U.V. absorption band (λmax., 2420A.) appeared during the conversion with acid; penillic acids are known to exhibit a band at about this region. In later experiments the quality of material at this point was much poorer, apparently owing to the changed composition of the corn-steep liquor and possibly to other factors as well; some changes, such as the use of ether instead of chloroform, and $NaHCO_3$ instead of $Ba(OH)_2$, are shown in the accompanying schematic concentration of six batches, a total of 240 l. culture solution.

Each batch (120,000—160,000 units)
→ charcoal eluate
→ ether extract (*ca.* 4 l.; 80,000—85,000 units)
→ aqueous Na salt (500 c.c.)
→ ether extract (350 c.c.; *ca.* 26,000 units)
→ aqueous Na salt (20 c.c.)
→ lyophilically dried solid.

The bulked crude sodium salt from the six batches was chromatographed on a partition chromatogram. The method is essentially the "chemical chromatographic" method briefly described by Catch, Cook and Heilbron (1942, 1943) as modified by Levi and Terjesen (1946). An ethereal extract (70 c.c., 140,000 units) of the penicillin was run on to a column (length 25 cm.) of silica-gel (65 g.) loaded with water and potassium phosphate buffer pH 6·50. The chromatogram was developed with ether (1400 c.c.) at 0° C. Fractions were extracted with neutral buffer (50 c.c.) and assayed together with a neutral extract of the eluate. Results are given in Table I. The material from fractions 9–12 and the eluate were combined and rechromatographed as before but at pH 6·70 with the results given in Table II.

Fractions 5–8 were extracted with ether (100 c.c.) at pH 2·4 and the penicillin recovered by titration of the ethereal solution after shaking with water (10 c.c.) with 0·1 N NaOH. The aqueous sodium salt was lyophilically dried.

The salt (150 mg., *ca.* 60,000 units) in water (20 c.c.) was acidified to pH 2·4 with 0·5 N HCl (*ca.* 1 c.c.). A small amount of yellow pigment was removed by

TABLE I.—*Chromatography of a Penicillin Concentrate obtained from* Aspergillus parasiticus.

Fraction.	Activity (Oxford units).	Depth of zone (cm.).
1	2,000	2
2		2
3		2
4		2
5	1,500	2
6		2
7		2
8		2
9	1,200	2
10	5,000	2
11	50,000	1·5
12	31,250	1·5
Eluate	17,500	—

TABLE II.—*Chromatography of Fractions* 9–12 *and Eluate* (*Table I*).

Fraction.	Activity (Oxford units).	Depth of zone (cm.).
1	2,000	2
2	<1,000	2
3	<1,000	2
4	<1,000	2
5	37,500	2
6	75,000	2
7	37,500	1·5
8	3,750	1·5
9	2,000	1·5
10	1,500	1·5
11	3,000	1·5
12	5,500	1·5
13	5,500	2
Eluate	5,000	—

extracting thoroughly with ether (5 × 15 c.c.). The aqueous solution was extracted with butanol (2 × 10 c.c.), and the extract evaporated *in vacuo* at < 30° C. to 1 c.c. and cooled to 4° C. overnight. Penillic-II acid, m.p. 187–188·5° C. (decomp. with mercaptan-like odour) separated in colourless rhombs (10·6 mg.); it was recrystallized by dissolving in dilute aqueous $NaHCO_3$ and bringing to pH 2. with *very dilute* HCl. (Found: C, 58·0; H, 6·1 per cent. Calc. for $C_{16}H_{18}O_4N_2S$; C, 57·5; H, 5·4 per cent). The melting point of a mixture with authentic penillic-II acid (m.p. 192° C.) was 188° C. A further crop (6·4 mg., m.p. 188–189° C.) was obtained from the mother-liquor. It was crystallographically identical with penillic-II acid (see above).

DISCUSSION.

The experiments described show that there is no doubt that the main antibiotic produced by *A. parasiticus* under the conditions described is penicillin-II, and the distinctive name "parasiticin" is no longer justified.

The production of a penicillin by still another *Aspergillus* is no longer very remarkable (Johns, Philpot and Pollock, 1946), but the present result has greater interest in relation to the small number of instances where the penicillins produced by *Aspergilli* have been identified. Gigantic acid (Philpot, 1943) has been identified (Dr. D. Crowfoot, Oxford, private communication) with dihydropenicillin-I which has been fully studied in these laboratories; gigantic acid, produced by *Aspergillus giganteus*, gave a small yield of dihydropenillic-I acid as obtained from *P. notatum*. On the other hand "flavicidin," a penicillin from *A. flavus* has been shown to be isomeric with penicillin-I (Fried, Koerber and Wintersteiner, 1946), whereas the penicillin produced by certain other *Aspergilli* has been thought on the basis of biochemical and physical behaviour to be "authentic penicillin" (presumably penicillin-II) (Foster and Karow, 1945). The versatility of the *Aspergilli* in producing penicillins of different structures seems therefore to be comparable with that of the *Penicillia*.

SUMMARY.

" Parasiticin," an antibiotic produced under selected conditions by *Aspergillus parasiticus*, has been shown on biological and chemical evidence to be largely, if not wholly, penicillin-II (–G) (benzylpenicillin) ; the distinctive name should be abandoned.

We thank Sir Ian Heilbron, D.S.O., F.R.S., for his interest and encouragement, and Miss M. S. Lacey, D.Sc., for antibacterial assays.

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THE PROZONE PHENOMENON IN RELATION TO THE AGGLUTINABILITY OF *S. TYPHI*.

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In a recent paper on the effect of heat and other forms of treatment on the somatic agglutinability of *S. typhi*, Hayes (1947*a*) showed that normal rabbit serum and human serum, collected after an attack of typhoid fever or inoculation with T.A.B. vaccine, may agglutinate suspensions of *S. typhi* strain 901/*O* to a high titre, while the same suspension, after boiling for a short time, is agglutinated either to a low titre or not at all. When tested against high titre immune rabbit sera such unheated and heated suspensions showed little difference in agglutinability. Similar results were obtained with a proportion of freshly isolated strains of *S. typhi* after removal of their Vi antigen, and evidence was produced which suggested that loss of somatic agglutinability by normal rabbit and human sera after heating or treatment with certain concentrations of alcohol was a discontinuous variation induced by artificial culture. Various theories which, it was

thought, might explain the mechanism of this phenomenon were discussed. Among these, one of the most probable was that heating so altered the physical properties of the cell that colloid adsorbed from the serum protected the organism from antibody action. In the work to be described, this suggestion has been investigated in detail.

Two distinct types of prozone have previously been described. For purposes of subsequent discussion these will be called A and B respectively. Prozone A occurs with unheated serum. Part of its mechanism was elucidated by White (1931), who showed that fresh normal rat or rabbit serum had the power of inhibiting the specific somatic agglutination of salmonellae. Inhibition did not occur in the presence of 1·7 per cent NaCl or higher concentrations, nor did it occur if the serum was heated at 55° C. or over. Supported by a more stable factor, complement was almost certainly an essential agent in this phenomenon. Prozone B occurs with sera which have been heated. This type was first described by Streng and confirmed by Shibley (1929). Its probable mechanism was elucidated by Bawden and Kleczkowski (1942), who demonstrated that heating induced combination between the albumins of the serum and antibody globulins, the resulting complex combining with antigen without precipitation.

METHODS.

1. Suspensions of *S. typhi* strain 901/*O* were prepared as follows: The strain was plated and several smooth colonies were selected. Each was inoculated on to two agar slopes. After overnight incubation one of each pair of cultures was refrigerated. The growth from the other was suspended in 1 : 2500 HgI_2–0·45 per cent NaCl solution, and the opacity of the suspension was adjusted to be equivalent to that of a suspension of 6000 $\times$ 10^6 *Bact. coli* per ml. Each suspension was then divided into two portions, one of which was heated in a boiling water-bath for 15 minutes, the other being untreated. Unheated and heated suspensions from each colony were then tested for sensitivity to agglutination by a sample of the human serum used throughout this research. The suspension showing the greatest and most clear-cut fall in sensitivity to agglutination after heating was considered most suitable for test. Its duplicate culture was suspended in broth and seeded to a large surface of nutrient agar. After overnight incubation the growth was harvested and a suspension was made and standardized as before.

2. The serum used was that of the author who had received his last "maintenance" dose of T.A.B. vaccine about two years prior to the commencement of the investigation. This serum recommended itself by its consistently high titre for unheated, and low or absent titre for heated, T/901/*O* suspension, as well as by its constant availability. Samples of serum were used as soon as possible after withdrawal of the blood, and without the addition of preservative.

3. Since the investigation was primarily concerned with the qualitative alterations induced in T/901/*O* by heating, neither the suspensions nor the samples of serum used were serologically standardized. It is doubtful, in any case, whether the serological standardization of suspensions against rabbit antisera bears any relation to the results obtained with human sera on account of the difference of relative agglutinin response of the rabbit and man to the various somatic antigens of *S. typhi* (Hayes, 1947*b*). The more logical procedure of standardization against human sera would have been invalid in this case, since

the properties of both serum and suspension were under investigation. Several batches of suspension and many samples of serum were employed during the course of the work. Differences in titre apparent in the protocols of different experiments may, therefore, be due to variation either in the sensitivity of various batches of suspension or to change in the titre of the serum. The same samples of serum and suspension were, of course, used throughout the course of any one experiment.

4. Serum dilutions were made in 1 ml. volumes in 1 : 2500 HgI_2–NaCl solution, buffered at pH 7·4. One drop of suspension was added to each tube. Results were read after two hours at 37° C. and 18 to 24 hours at room temperature. The titre was taken as the highest dilution of serum which did not show a discrete spot of unagglutinated bacteria. Sometimes a small or diffuse spot surrounded by aggregates of agglutinated bacteria occurred in this dilution. Such results are recorded as "$\pm$" or "Tr." depending on the relative degree of agglutination or spotting when compared with a control tube containing no serum. In all cases the titre is recorded as the reciprocal of the serum dilution concerned.

EXPERIMENTAL FINDINGS.

The effect of increasing the concentration of NaCl from 0·85 per cent to 3·4 per cent on the agglutinability of unheated and heated suspensions of T/901/*O* by human serum, as well as by normal and immune rabbit sera, was investigated. In 0·85 per cent NaCl a sample of human serum showed a titre of 1280 against unheated suspension and of 5 against heated suspension; the titres of the same serum in 3·4 per cent NaCl were 640 and 160 respectively. Similarly, the presence of 3·4 per cent NaCl slightly lowered the titre of normal rabbit serum from 640 Tr to 320 against unheated suspension, but raised that against heated suspension from 20 Tr to 160. Diluted, high titre, homologous rabbit antiserum agglutinated unheated suspension to a titre of 320 and heated suspension to a titre of 160_+. These titres remained the same irrespective of whether 0·85 or 3·4 per cent NaCl was used as diluent.

A recently isolated strain of *S. typhi* (T/16/46) which was deficient in, though not devoid of, Vi antigen was similarly tested against human serum after treatment with absolute alcohol to destroy flagellar agglutinability. With this strain, the use of 3·4 per cent instead of 0·85 per cent NaCl as diluent increased the titre against heated suspension from less than 5 to 1280 Tr, while that against unheated suspension was raised from 1280 to 2560.

Increase in NaCl concentration from 0·85 to 3·4 per cent, therefore, raises the agglutinability of heated *S. typhi* suspension to a level comparable to, though usually slightly lower than, that of unheated suspension. In other words, the relative sensitivities of heated and unheated suspensions to human and normal rabbit serum in 3·4 per cent NaCl solution are similar to those given by high titre immune rabbit serum in 0·85 per cent NaCl.

Unlike the inhibitory properties ascribed by White to fresh rat and rabbit serum, inhibition of agglutination of heated T/901/*O* suspension was increased rather than abolished by heating the serum. The effect of concentrations of NaCl varying from 0·1 to 13·6 per cent on the agglutination of unheated and heated suspensions by fresh human serum, unheated and heated at 58° C. for 30 minutes, is shown in Table I.

TABLE I.—*The Effect of Various NaCl Concentrations on the Agglutinability of* S. typhi *Strain* 901/*O by Human Serum, Unheated and Heated at* 58° *C. for* 30 *minutes.*

NaCl concentration (%)	Suspension T/901/*O*.	Titre of— Unheated serum. Untreated.	Unheated serum. Heated.	Heated serum. Heated.
	13·6	160	160	160 Tr
	6·8	160	80	80 Tr
	3·4	160	80 Tr	20
	1·7	320±	20	<10
	0·85	320	<5	<5
	0·42	320	5	10±
	0·21	320	20±	<10
	0·1	320	20	<10

The concentration of NaCl producing maximum inhibition of agglutination of heated suspension is, therefore, 0·85 per cent. With lower concentrations inhibition is less marked, while with higher concentrations the agglutinability of unheated and heated suspensions tends to approximate. Since heating did not abolish inhibition, it was clear that the phenomenon was different from the complementoid effect described by White.

In order to determine whether any serum component might be responsible for inhibition, the serum was fractionated by half saturation with neutral ammonium sulphate. The precipitated globulin was washed in 50 per cent ammonium sulphate and redissolved in 0·85 per cent NaCl. Both this and the supernatant albumin fraction were dialysed against frequent changes of 0·85 per cent NaCl at 4° C. until free from ammonium salts, as judged by absence of colour with Nessler's reagent. The volume of each fraction was then adjusted to be equivalent to some low dilution of the original serum—usually 1 : 2 or 1 : 2½. Fractions so obtained were tested alone and in combination against unheated and heated suspensions of T/901/*O*. Results are shown in Table II.

TABLE II.—*The Behaviour of Globulin and Albumin Fractions of Human Serum in the Agglutination of* S. typhi *Strain* 901/*O*.

Serum fraction.	Titre of serum fraction *v.* T/901/*O*. Unheated suspension.	Heated suspension.	NaCl conc. (%).
Untreated serum	1280 Tr	<10	0·85
	640	160 Tr	3·4
Globulin	640±	80	0·85
	..	160 Tr	3·4
Albumin	..	<10	0·85
	..	<10	3·4
Globulin in 1 : 10 albumin	..	10	0·85
	..	160 Tr	3·4

Several similar fractionation experiments were performed both with different samples of human serum, and with a normal rabbit serum having titres of 640

and 40± for unheated and heated T/901/*O* suspensions respectively. In every case the globulin fraction alone agglutinated heated suspension to approximately ½ or ¼ its titre against unheated suspension, behaving in much the same manner as whole serum in the presence of high saline concentration. Reconstitution of the albumin and globulin fractions, however, did not always re-establish the inhibitory effect shown by the unfractionated serum, although inhibition invariably resulted when the concentration of the albumin fraction was greater than that of the globulin. For example, the titre of one sample of human serum against heated suspension was less than 10, of the globulin fraction 160, of the albumin fraction less than 10 and of the reconstituted albumin and globulin fractions 160 Tr, but with no agglutination in the 1 : 10 dilution. On the other hand, globulin fraction diluted 1 : 20 in 1 : 5 and 1 : 10 albumin solution gave no agglutination.

It seemed probable that inhibition of agglutination of heated suspension was due to interference by some part of the albumin fraction of serum. The failure to reproduce the phenomenon consistently and completely with reconstituted albumin-globulin mixture was probably due to alteration in the colloidal properties of one or both components during fractionation. That such alteration does occur is evident from the fact that both fractions tend to precipitate slightly during dialysis against NaCl in the cold, the globulin solution becoming faintly opalescent as well as showing some deposit. Albumin solution appears to exercise a stabilizing influence on dialysed globulin. When mixtures of both fractions in varying proportions were being prepared for test, it was noticed that a 1 : 5 dilution of dialysed globulin in NaCl was opalescent, but became increasingly clear when the same dilution was prepared in albumin in concentrations of from 1 : 80 to 1 : 5 in NaCl.

In order to study the inhibitory properties of serum, uninfluenced by ammonium sulphate precipitation, a sample of human serum was divided into two parts from each of which all trace of T/901/*O* agglutinin was removed by absorption, one with unheated and the other with heated suspension (90×10^9 organisms for each ml. serum). Varying dilutions of each of these absorbed sera were made in 1 : 20 dialysed globulin from the same serum before absorption, and the mixtures were tested for agglutinating action against heated suspension. Results are given in Table III.

It should be mentioned that the globulin fraction alone has never shown zoning in any concentration, but that the quality of agglutination produced by it is much finer in all dilutions than that produced by unfractionated serum. Whole serum, devoid of agglutinin and also, presumably, of complement, is capable, therefore, not only of suppressing agglutination by globulin alone, but also of intensifying agglutination when present in high concentration. This latter effect may be similar in nature to the " conglutination reaction " described by Wiener (1945).

In order to determine whether the proportional relationship between the agglutinin-free components of serum and the antibody globulin was relevant to the development of zoning, the effect of varying dilutions of human serum, completely absorbed with heated suspension, on the agglutinating action of varying dilutions of globulin fraction was studied. Results are shown in Table IV.

It was found that every dilution of agglutinin-free serum was able completely to inhibit agglutination by its equivalent or higher globulin dilution. In dilutions of absorbed serum higher than 1 : 20, however, excess of globulin was capable

TABLE III.—*Effect of Varying Dilutions of Human Serum Absorbed with either Heated or Unheated T/901/O Suspension, on the Agglutinating Action of Globulin Fraction from the Same Serum.*

Dilution of absorbed serum.†	Agglutination of heated T/901/*O* suspension (in 0·85 per cent NaCl). 1 : 20* globulin fraction in varying dilutions of whole serum absorbed T/901/*O*.	
	Serum absorbed with unheated suspension.	Serum absorbed with heated suspension.
1 : 5	++‡	++‡
1 : 10	+	−
1 : 20	+	−
1 : 40	−	±
1 : 80	−	+
1 : 160	+	+
1 : 320	+	+

* Titre of globulin fraction alone *v.* heated T/901/*O*, 160.
† Titre of absorbed serum alone *v.* untreated T/901/*O*, less than 5.
‡ Very marked agglutination.

TABLE IV.—*The Effect on Agglutination of Different Mixtures of Human Serum, Absorbed with Heated T/901/O Suspension, and Globulin Fraction from the Same Serum.*

Dilution of absorbed serum.	Agglutination of T/901/*O* (heated) (0·85 per cent NaCl). Dilution of globulin fraction.						
	5	10	20	40	80	160	320
10	−	−	−	−	−	−	−
20	−	−	−	−	−	−	−
40	+	±	±	−	−	−	−
80	+	+	+	+	−	−	−
160	+	+	+	+	+	−	−
320	+	+	+	+	+	+	−
No serum	+	+	+	+	+	+	−

of overcoming this inhibitory effect. Although mixtures constituted in this way are not strictly comparable with the untreated whole serum, since their content of globulin is higher, nevertheless their behaviour is the same, agglutination being inhibited in every dilution up to the true titre of the globulin fraction.

A similar experiment was performed with serum absorbed with unheated instead of heated suspension. Sera rendered agglutinin-free by absorption with unheated and heated suspensions had given different results when mixed in varying concentrations with one dilution of globulin fraction, despite the fact that the sera were initially identical and the same number of organisms per volume was used for absorption in both cases (Table III). In this experiment only the 1 : 40 dilution of absorbed serum inhibited agglutination of heated suspension by globulin in all concentrations tested. The 1 : 10 dilution failed to inhibit agglutination at all, the 1 : 20 dilution inhibited at both ends of the range of globulin concentrations but not in the middle, while the 1 : 80 and 1 : 160 dilutions only effected an apparent lowering of the titre

of the globulin fraction. Thus with this agglutinin-free serum a pronounced inhibitory optimal occurred. The experiment was not repeated. A similar kind of result has occasionally been noticed when titrating whole sera against heated T/901/*O* suspension, some agglutination occurring in high concentrations of serum, followed by a zone, so that the apparent titre of the serum is markedly lower than its true titre as shown by titration in 3·4 per cent NaCl solution. Perhaps the zone pattern most frequently seen with human sera and heated suspension is the occurrence of one tube showing " trace " or " $\pm$ " agglutination in the middle of a series of serum dilutions showing discrete spots. This tube usually corresponds to the true titre, or one half of the true titre, of the serum.

The Mechanism of Inhibition.

Evidence has been produced which strongly suggests that the absolute or relative inagglutinability of heated *S. typhi* suspensions by human or normal rabbit sera is due to interference in the reaction between antibody globulin and the cell surfaces by the albumin moiety of serum. Two broad theories can be postulated to explain such interference :

(1) A primary non-specific adsorption of " albumin fraction " by the cell which prevents the fixation of agglutinin on the cell surface.

(2) A secondary adsorption of " albumin fraction " by the agglutinin-cell complexes which inhibits aggregation.

Experiments were performed to determine which of these two mechanisms was involved.

It was found that inhibition of agglutination in 0·85 per cent NaCl was unaffected by prior contact of both heated suspension and human serum with 3·4 per cent NaCl. Moreover, heated suspensions which were left in contact with a 1 : 2 dilution of dialysed albumin, or with a concentration of agglutinin-free serum which produced inhibition in a control experiment, and then washed, were agglutinated normally in 0·85 per cent NaCl by dilutions of antibody globulin. High concentrations of NaCl, therefore, did not abolish inhibition by imposing any irreversible change on either suspension or serum, while the inhibitory substance, if adsorbed at all by unsensitised suspension, was so loosely bound as to be readily eluted by washing.

It was also found that heated suspension, although inagglutinable, absorbed precisely the same amount of specific agglutinin from human serum as did an equivalent volume of the unheated suspension.

This experiment was repeated twice, using different samples of serum and batches of suspension, with the same results. In these later experiments the absorbing suspensions, after decanting the absorbed serum, were gently washed three times in small volumes of either 0·85 or 3·4 per cent NaCl. The washings were pooled and tested for eluted agglutinin. No agglutinin was detected. Despite the fact that agglutinin had clearly been bound and had not been eluted, the sensitized cells of both unheated and heated suspension showed very variable agglutination in either 0·85 or 3·4 per cent NaCl. In one experiment the sensitized, unheated bacteria were unstable, and in another stable in both NaCl concentrations. In all three experiments, the sensitized, heated bacteria were quite inagglutinable in 0·85 per cent NaCl and, in two, in 3·4 per cent NaCl also. The reason for these irregularities is not known.

It has been shown that heated suspensions are not rendered inagglutinable by antibody globulin by prior treatment with albumin solution or with agglutinin-free serum known to produce inhibition of agglutination by antibody globulin, but are nevertheless capable, without being agglutinated, of absorbing and retaining homologous *O* agglutinin from human serum to the same extent as agglutinable, unheated suspensions. It seems probable, therefore, that the inhibitory "albumin fraction" only becomes bound to the cell either during or after its union with antibody.

The essential determinant of inhibition is obviously the alteration produced in the surface of the cell by heat. The effect on the development of inagglutinability of growing cultures at 37° C. and 24° C., of harvesting during the logarithmic and stationary phases of growth and of employing different pHs during the heating process have been studied. Inagglutinability after heating is most clearly shown by logarithmic phase cultures and by cultures grown overnight at 24° C. The effect of cultivation at 24° C. is especially marked with freshly isolated strains of *S. typhi*. At this temperature the development of Vi antigen is suppressed (Felix, Bhatnagar and Pitt, 1934), so that the marked agglutination of the unheated suspension forms a striking contrast to the discrete spots shown by the same suspension after heating.

The effect of pH during heating was investigated by dividing T/901/*O* suspension into four parts, centrifuging, and resuspending the deposit from each in 0·85 per cent NaCl buffered at different pH. Each suspension was heated in a boiling water bath for 15 minutes, washed and resuspended to its original density in 0·85 per cent NaCl buffered at pH 7·2, and tested against dilutions of human serum in both 0·85 and 3·4 per cent NaCl. Results are given in Table V.

TABLE V.—*The Effect of pH during Heating on the Agglutinability of T/901/O Suspension by Human Serum.*

Suspension heated at pH.	Agglutination by human serum in dilutions of —							NaCl conc. (%).
	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	
6·6	—	—	—	Tr	Tr	—	—	0·85
	+	+	++	++	++	+	—	3·4
7·0	—	—	—	—	—	—	—	0·85
	+	+	+	+	+	±	—	3·4
7·4	—	—	—	—	—	—	—	0·85
	Tr	Tr	Tr	+	±	Tr	—	3·4
7·8	—	—	—	—	—	—	—	0·85
	Tr	Tr	+	+	+	Tr	—	3·4

The development of inagglutinability is most marked when the suspension is heated at pH 7·0 or over, though as the pH increases beyond 7·0 the abolition of inhibition by 3·4 per cent NaCl is less clearly shown. Conversely, with the suspension heated at pH 6·6, zoning is not clean-cut with certain serum dilutions, but the development of agglutination in 3·4 per cent NaCl is most marked. It was observed that the suspension heated at pH 6·6 deposited readily on centrifuging, leaving a clear supernatant fluid. As the pH increased, deposition became increasingly difficult. This indicates that heating at pH above neutrality increases the hydrophile tendencies of the suspension—a factor which may play a direct role in the stability of heated suspensions to agglutination.

Other Types of Inhibition.

Prozones A and B, occurring with unheated and heated human serum respectively, have also been observed with unheated *O* suspensions of T/901 and other strains of *S. typhi*. In prozone A, inhibition of agglutination commonly did not extend beyond 1 : 10 or 1 : 20 dilutions of the serum and was rare in dilutions above 1 : 40. Zoning was abolished by heating the serum, or when dilutions of the unheated serum were made in NaCl concentrations of 1·7 or higher. It was not induced by agglutinin-free serum. Prozones A and B can be demonstrated with the same sample of serum. For example, one sample of human serum was tested against unheated suspension before heating and after heating at 58° C. for 30, 60, 90, 105 and 140 minutes. With unheated serum zoning occurred in the 1 : 10 dilution. This zone was absent from all dilutions of serum heated for 30 minutes, clean agglutination occurring in every tube. After 60 minutes' heating only partial agglutination occurred in the 1 : 10 dilution, while after more prolonged heating inhibition of agglutination in this dilution was complete. This type of inhibition has also been demonstrated with *Br. abortus* suspension and a sample of serum from a patient suffering from *Br. abortus* infection (Dr. F. S. Stewart and Dr. J. D. McKeever—personal communication, 1946). Zoning occurred when the agglutination test was put up at 55° C., but not at 37° C. After the serum had been heated at 55° C. for one hour, zoning occurred at 37° C.

DISCUSSION.

A new type of prozone is described which differs from prozones A and B. This type will provisionally be called prozone C. It occurs when heated *S. typhi O* suspension is mixed with unheated serum. Inhibition is so potent that a serum having a titre of 1280 against unheated suspension may show no agglutination of heated suspension in any dilution, although the sensitivity of the suspension to agglutination by high titre, homologous rabbit antiserum is usually only reduced to between two and four times by heating, and may not be reduced at all when tested against *S. enteritidis O* antisera (Hayes, 1946). Evidence has been produced which suggests that inhibition is due to the incorporation of some fraction of serum albumin in the cell-agglutinin complex, although albumin is not adsorbed by the cell in the absence of antibody. The mechanism of prozone C, therefore, probably differs from prozone B only in the fact that the determinant of union of both antibody and albumin on the cell surface is the cell itself rather than the serum.

Ehrlich attributed zoning to the presence in the serum of agglutinoid, a kind of degraded agglutinin which had retained its haptophore but lost its zymophore grouping, so that it could combine strongly with homologous antigen without being able to produce agglutination. Shibley found confirmation of a modified agglutinoid theory, in his work on prozone B, by demonstrating that antibody rendered incapable of agglutinating by heat still possessed high combining power, and could be specifically absorbed from the serum. He postulated that the effect of heat was to change agglutinin into agglutinoid. The work of Bawden and Kleczkowski showed that agglutinoid was probably a complex of albumin and agglutinin rather than agglutinin which had become degraded. The demonstration that alteration in the antigen alone can produce a prozone, the mechanism of

which is probably similar to that shown by Shibley, removes the necessity for postulating the presence of agglutinoid at all since, in prozone C, the serum has not been altered in any way and does not produce a zone of this type against unheated suspension. A similar conclusion has been reached by Jenkins (1946), who showed that *O* suspensions of *S. typhi* which had been treated with 40 per cent neutralized pyridine gave prozones with homologous Oxford *O* antisera, though a prozone did not develop if the suspension was heated either before or after the pyridine treatment. He infers that prozone is determined by the state of the antigen, and that "agglutinoids are unlikely to have anything to do with the results obtained." Nevertheless he states that prozones develop best at about 57° C. and, though still present at 50° C., are not so marked. In the present work prozone C was observed with sera which had never been subjected to temperatures higher than 37° C.

Apart from their practical importance, the chief interest of prozones lies in the light they throw on the mechanism of agglutination and other antigen-antibody reactions. While there is general agreement on the specificity of the primary union of antigen and antibody in agglutination and precipitation, controversy still exists concerning the so-called "second stage" of these reactions —the aggregation of the antigen-antibody complexes. The upholders of specificity contend that "by a repetition of specific acts of combination, larger and larger molecular complexes are elaborated" (Lanni, 1946), so that ultimately macroscopic aggregates are formed by a continuation of the initial union of the primary units of the reactants. This concept is in accordance with Marrack's "lattice" hypothesis, which connotes at least two valencies for antibody in order that the primary complexes may continue to unite specifically, and implies that prozones are due to too great divergence of the concentrations of antigen and antibody from equivalence except, perhaps, in cases where very high concentrations of serum are employed. The opponents of specificity maintain that antigen becomes specifically coated with antibody globulin which is denatured in the process, so that the complex behaves like a particle of denatured globulin and is precipitable by salt (Shibley, 1924, 1926). Eagle (1930) suggests that the hydrophile groupings of antibody globulin are largely associated with its reactive groupings and are masked by union with antigen, so that balance is upset in favour of the hydrophobe groupings. This theory does not require that antibody should be multivalent, but fails to explain why, when two different reacting systems of morphologically and antigenically distinct bacteria and their antibodies are mixed, the resulting aggregates are homogeneous of one type or the other (Topley, Wilson and Duncan, 1935)—an experimental finding which greatly favours a "lattice" mechanism for agglutination, though results have not always been consistent, especially in the precipitin reaction.

Any theory of antigen-antibody reaction must be capable of explaining why, under certain conditions, the reaction fails to manifest itself. Prozones resulting from heated sera might be explained on the "lattice" theory by supposing that heat transformed multivalent into monovalent antibody by forcing the surplus valencies into combination with albumin, though it is difficult to conceive why all the valencies should not be so affected. Such a theory is not applicable to prozone C. For the masking of secondary valencies in this case, molecules of albumin would have to be superimposed on the already combined globulin and, therefore, out of contact with the cell which, solely, had determined their adsorption. If it is supposed that the specific groupings of the cell, which combine with

antibody, do not occupy its entire surface, it is more probable that molecules of antibody and " albumin fraction " both combine directly with the cell, and that agglutination is prevented, in accordance with Eagle's theory, by an excess of hydrophilic groupings supplied by the salt-stable " albumin fraction." This would also more readily explain the reversibility of the zone in high concentrations of saline. The homology of the particles in aggregates, resulting from mixture of two antigen-antibody systems reacting at the same velocity in one tube, by no means excludes a serologically non-specific mechanism of aggregation. If it is assumed, on the ground that non-specific serum complexes can apparently be adsorbed on to the surfaces of bacteria without affecting their agglutinin-binding capacity, that antigen is not completely covered by antibody when the two combine, then it is clear that specific aggregation might be explained by the different surface potentials of antigen-antibody systems of different type.

The patterns of the prozones and prozonal lags demonstrated by Jenkins (1946) with pyridine treated somatic suspensions were sufficiently specific to distinguish between the somatic structures of *S. typhi* and *S. enteritidis*, and of *S. paratyphi B* and *S. typhimurium*. Their behaviour, although induced solely by treatment of the antigen, simulated that usually associated with the precipitin reaction in which the occurrence of optimal proportions and zoning is universally attributed to a mechanism of " lattice " type. Jenkins also showed that pure dioxan or pyridine flagellar extracts developed prozones much more readily than *H* antigens, and suggested that their behaviour might indicate that a precipitin reaction was occurring with an antigen in solution. Pure antibody, eluted from flagellar suspensions which had been mixed with excess antiserum and then washed free of surplus antibody, failed to show any prozone or prozonal lag with pyridine or dioxan extracts of flagella. This suggests that the prozonal patterns shown by these extracts and rabbit antiserum cannot be explained on the " lattice " theory, but are due to interference by some substance absent from pure antibody.

Reference to Table III of the present paper shows that the highest concentration of serologically non-specific substance in human serum does not necessarily exercise the most marked inhibitory effect on agglutination. When serum, from which all agglutinin had been removed by absorption with unheated suspension, was tested in various dilutions with 1 : 20 antibody globulin, only the 1 : 40 and 1 : 80 dilutions inhibited agglutination, higher and lower dilutions allowing the second stage of the reaction to occur. When the same serum was absorbed with heated suspension under precisely the same conditions, however, the inhibitory zone was shifted two tubes down, presumably due to partial absorption of inhibitory substance by the cell-agglutinin complex. Again, the results of testing suspensions heated at different pH against human serum in two concentrations of sodium chloride (Table V) show the development of an agglutination optimum in 3·4 per cent sodium chloride which is clearly correlated with the development of prozone C in 0·85 per cent sodium chloride.

It has been mentioned that the only change discernible in suspensions after heating was an increased stability in sodium chloride. Stability was greater at pH which induced a more marked prozone effect. If this hydrophile tendency was the determinant of ability to adsorb what, for the sake of simplicity, has been called the " albumin fraction " which is apparently responsible for the development of prozones and agglutination optima, this mechanism should operate most efficiently if the antigen is in its most hydrophilic state—that is, in solution in a precipitin reaction. Since the amount of albumin in serum (4·5 g. per cent)

is nearly three times that of globulin (1·7 g. per cent), it follows that any dilution of serum containing sufficient globulin to produce agglutination or precipitation will contain a greater proportion of albumin.

It is submitted that the phenomena of optimal proportions in the precipitin and agglutination reactions might prove capable of complete explanation on the basis of a two-stage reaction in which " albumin fraction " determined inhibition. Such a concept, for example, would more readily account for the disparity between constant antigen and constant antibody optimal ratios than does the " lattice " theory, which supposes only two reactants, since the inhibitory optima for " albumin fraction " may clearly differ widely, depending on whether the serum is kept constant or is varied.

SUMMARY.

1. A new type of prozone obtained with human serum and somatic suspensions of *S. typhi* is described.

2. The development of this prozone is determined solely by treatment of the suspension with heat, which does not affect either the specificity of the cell surface or its power of combination with antibody.

3. It is probable that this prozone is due to the adsorption by the cell-agglutinin complex of part of the albumin fraction of serum.

4. The theoretical implications of prozones determined by alteration in the physical state of the antigen alone are discussed in detail.

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TITRATION OF *CL. WELCHII* EPSILON TOXINS AND ANTITOXIN.

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THE principal toxin produced by *Cl. welchii* Wilsdon Type D was termed epsilon by Glenny, Barr, Jones, Dalling and Ross (1933), who described the toxin as necrotic and lethal. At the time of publication this toxin had not been closely studied; we can now state that ε toxin forms necrotic areas when injected intracutaneously into rabbits and guinea-pigs, is lethal to mice and rabbits injected intravenously, and produces extensive oedema and death when given subcutaneously to guinea-pigs. The phenomenon, reported by Bosworth and Glover (1934–1935), of increase in toxicity of *Cl. welchii* Type D filtrates after a short exposure to trypsin greatly increased the interest of ε toxin. Further interest has been stimulated by the conclusion, reached by Turner and Rodwell (1943), that most Type D filtrates, after a short period of growth, contain, in addition to some toxin, an almost atoxic, relatively thermostable precursor referred to as a proto-toxin, which has the same combining power as toxin, and which can be converted into toxin by various proteolytic enzymes. The total amount of antigen, proto-toxin and toxin produced in a broth culture may almost reach a maximum in 6 to 24 hours; during further incubation much of the proto-toxin is changed to toxin by the action of enzymes produced by the organism, but filtrates from 4 or 5 days' growth still contain some unactivated proto-toxin since further activation can be obtained by the action of trypsin. The observation by Glenny (1936) that toxins increase in toxicity when kept in phosphate buffer solution can be explained by the improvement in the conditions for the action of the bacterial enzyme.

Methods of testing toxins can be divided into two main divisions—tests for toxicity and tests for power of combination with antitoxin. The former cannot be regarded as accurate unless many animals are used, but any form of combining power tests yields results with an accuracy of well within 10 per cent for an expenditure of few animals. Toxicity tests are made without reference to any arbitrary standard, and apart from variation of susceptibility of the animals used and the conditions under which they are kept, results obtained in different laboratories can be compared directly. A measurement of toxicity, however, is of very little practical use, except to determine the extent of change of proto-toxin into toxin during growth or after treatment of a filtrate with trypsin. Combining power tests on the other hand are of special importance in assessing the total amount of antigen present, whether in the form of toxin, or toxoid, and in the case of epsilon toxin of proto-toxin as well. An arbitrarily chosen antitoxin must be used, and so no comparison can be made between the results of different workers using different standards. The unit of antitoxin used in these laboratories was fixed in 1933, but unfortunately we have not yet had an opportunity of comparing our unit with that adopted by Turner and Rodwell (1943).

The flocculation reaction.

Mixtures of unconcentrated antitoxic serum and untreated toxin show several zones of flocculation, and it is difficult to determine which is the specific zone. Antitoxin refined by the pepsin process when mixed with untreated toxin gives a definite zone of flocculation with an optimal point bearing a definite relation to *in vivo* end points, but non-specific flocculation also occurs, and can be recognized by its rapidity (less than 15 minutes under our conditions of test), or by the appearance of flocculation in all mixtures with excess toxin.

Trypsin treated toxins flocculate specifically with unconcentrated or refined antitoxin, and can be used for all preliminary testing of antitoxin.

The Lf dose is determined against an arbitrarily chosen refined antitoxin by mixing a graded series of volumes of toxin with 10 units of antitoxin, heating the mixtures in a water bath at 50° C., and determining the volume of toxin equivalent to one unit in the mixture first to flocculate. It cannot always be assumed that, for any chosen standard antitoxin, the antitoxic value as measured by *in vivo* methods can be satisfactorily taken as equal to that determined by the flocculation method. Unsatisfactory results are obtained if the standard serum originally chosen is non avid, although, when once the *in vitro* unit has been established, subsequent standard sera can be used provided that they are compared with the original standard by the same method of testing. Trypsin activation of epsilon toxin so increases the toxicity that it can be assumed that an Lf mixture should be only just non-toxic to mice, i.e. the L+ dose should be practically equal to the Lf dose. Our original pepsin treated antitoxin fortunately fulfilled these requirements, so it is safe to assume that the antitoxin had a serum ratio of 1·0, and the values assigned to any toxin or antitoxin have a theoretical as well as practical basis.

From the chosen antitoxin the Lf dose of a toxin can be determined, and using this dose the flocculation value of any antitoxin can be measured. Mixtures of activated toxin with refined antitoxin usually flocculate in from 30 to 45 minutes; with natural serum flocculation may appear in one hour, but usually not until several hours have elapsed.

With a satisfactory flocculation reaction at our disposal, other methods of measuring combining power have assumed less importance, because flocculation involves all forms of antigens equally, while the end point reached in any animal test depends upon (1) the total amount of antigen, (2) the amount of active toxin present, (3) the relative affinity for antitoxin of toxin, proto-toxin and toxoid, and (4) the avidity of the standard antitoxin in use. In some circumstances the animal tests for combining power have a qualitative rather than a quantitative value, in that they show the specificity of the flocculation reaction, since local reactions produced in the skin of rabbits or guinea-pigs are usually so typical of epsilon toxin. This point is not so important in the case of trypsin treated filtrates because trypsin destroys most other toxins or reacting antigenic material. The titration of antitoxins to be used clinically should always be made finally by animal experiment to demonstrate that the antitoxin can prevent typical symptoms or death produced by the toxin.

Intravenous injection into mice—lethal dose (L.D. 50).

The dose under test is contained in 0·5 ml. by making use of the appropriate dilution; mice weighing between 18 and 20 g. are used. It is advisable to

keep mice under observation for 96 hours, although with the majority of toxins examined deaths rarely occur after 24 hours. We do not yet know the significance of later deaths which sometimes follow the injection of certain toxins, but the reason for such deaths following the injection of mixtures of toxin and antitoxin is considered later. When accurate results are not needed it is sufficient to use pairs of mice at each dose, and to confirm the result on a second occasion. The end point taken would be the calculated dose that would kill 2 out of 4 mice injected. Mice may die within 3 hours after the injection of 50 L.D. 50 doses. The average filtrate after 24 hours' growth contains about 50 L.D. per ml.; after activation with trypsin this increases to 3000.

Combining power; L+ in mice.

This is the smallest dose of toxin which mixed with one unit of antitoxin kills 50 per cent of mice injected within 72 hours. When trypsin activated toxin is used, very few mice surviving for 24 hours eventually die. The significance of later deaths with unactivated toxins is considered later. It is essential in all combining power tests that the test dose should contain a sufficient number of reacting doses whether L.D. for L+ tests or M.R.D. for Lr tests. It is necessary therefore to use multiples of the L+ dose when tests are made on unactivated toxin. In practice the amount of toxin in the mixture injected intravenously is 10 times the L+ dose of untreated toxin, and 1·0 or 2·5 times when trypsin activated toxin is used. The L+ dose of toxin is used to confirm the readings of a flocculation test upon a toxin or to confirm the antitoxic value of a serum. It must be emphasized that L+ determinations of short growth unactivated toxins are misleading. Upon activation by trypsin no more antigen is produced, but the L+ dose diminishes considerably.

Intracutaneous injection into rabbits.

Considerable individual variations occur among rabbits injected intracutaneously with 0·2 ml. of dilutions of toxin. It is our custom to inject rabbits two days before use with 1 test dose of toxin mixed with 20 per cent less antitoxin than that which is sufficient just to prevent necrosis in the majority of rabbits injected. Only those that show a necrotic reaction are accepted for test purposes. In such chosen rabbits a positive necrotic reaction is produced by the intracutaneous injection of 0·2 ml. of a dilution of about 1 in 500 of a short growth toxin or of a 1 in 50,000 of an activated short growth toxin, or a long growth toxin. After 24 hours the reaction is usually a rather diffuse flat-domed swelling about one inch in diameter, with a vaguely outlined yellowish necrosis in which are scattered small purplish haemorrhages. After 48 hours the centre of the swelling has become slightly concave, and is occupied by a sharply defined circular area of whitish yellow necrosis about 1 inch in diameter. The injection of half this necrotizing dose produces in 48 hours a well-defined mottled purplish area without definite necrosis. It is usually possible to determine within a few hours at which site of injection either of these reactions will occur; reliable readings are made at the end of 2 days.

Some but not all the accepted rabbits react to injections of one hundredth or less of the minimal necrotic dose; this reaction is a rather diffuse slightly raised red area. It is not yet known whether the failure of other rabbits to produce this non-necrotic lesion is due to the range of susceptibility accepted by us. It has

been noticed that toxins have a greater tendency to produce this reaction when tested shortly after preparation, and that much smaller fractions of the minimal necrotic dose of trypsin activated toxin produce this type of reaction ; the range from no reaction of any kind in susceptible rabbits to a definite necrotic reaction is considerably greater when activated toxins are used.

Minimal necrotic dose, M.N.D., is the least quantity of toxin which, when injected intracutaneously, will produce definite necrosis in a rabbit.

The Ln dose in rabbits is the smallest dose of toxin which, mixed with 1 unit of antitoxin, causes a necrotic reaction in 50 per cent of the rabbits injected. This test has the advantage over the mouse lethal test in that specificity of the reaction is at once evident. Once the Ln of a toxin is known it can be used to titrate the antitoxic value of sera. We use the expression M.N.D. and Ln in distinction from M.R.D. and Lr, because we may ultimately use the end point of non-necrotic reaction when more work has been done.

Intracutaneous injection into guinea-pigs.

In guinea-pigs injected intradermally with 0·2 ml. of a 1 in 10 dilution of an average ε toxin or with one hundredth of this amount of activated toxin, the reaction at 24 hours consists usually of one, but sometimes of several sharply cut white necroses, sometimes irregular in shape (occasionally with patches of purplish haemorrhagic mottling), surrounded by a wide flush about one inch in diameter ; the whole reaction is raised as a dome above the general surface of the skin. By 48 hours most of the swelling and the flush have disappeared ; the necrosis alone remains, but may appear slightly smaller than at 24 hours. This reaction is so typical of epsilon toxin that it can be used to give a fairly reliable guide to the presence, but not the absence of this toxin in material of which the quantity available is not sufficient to enable tests to be made by means of neutralization tests with appropriate specific antitoxins.

Combining power tests in guinea-pigs are often unreliable if unactivated toxin is used, possibly because sufficient reacting doses are not available. With activated toxin, however, the test is quite reliable both for the determination of the Lr dose of a toxin and for the titration of antitoxin.

Subcutaneous injection into guinea-pigs.

This method of testing has been used by us occasionally for experimental purposes, especially with a view to finding whether *Cl. welchii* Type D may produce some toxin other than α, ε, θ or K, but we have not yet had any significant results. The lethal dose for guinea-pigs is about 1·0 ml. for a short growth filtrate before activation and 0·02 to 0·05 ml. after activation.

Measures of toxicity and combining power before and after activation with trypsin.

In Table I we record measurements of a 24-hour filtrate before and after treatment with trypsin. The figures quoted for the three methods of determining toxicity are not accurately determined. A large number of tests are needed for accurate measurement, and the result does not justify this expenditure of animals. The three activation ratios here recorded are within 25 per cent of the average, but with many toxins tested by us the three ratios may range from half to double the mean unless many tests have been made. We are inclined to the opinion

TABLE I.—*Showing the Various Measurements for Toxicity and Combining Power of a Short Growth* Cl. welchii *Epsilon Toxin* (*NX*.324) *before and after Activation with Trypsin.*

Toxicity.	Volume in ml. Unactivated.	Activated.	Activation ratio.
L.D. in mice	0·034	0·00025	136
M.N.D. in rabbits	0·010	0·00006	167
M.R.D. in guinea-pigs	0·06	0·0006	100
Combining power.			**Activation index.**
Lf	0·066	0·066	1·00
L+ in mice	0·126	0·068	1·85
Ln in rabbits	0·10	0·072	1·39
Lr in guinea-pigs	..	0·11	..

that the M.N.D. in rabbits is a slightly better measure of activation ratio, because the specific type of reaction produced gives this method of testing toxicity an advantage over methods involving death of mice which might be non-specific, which outweighs the disadvantage of the subjective determination of a positive reaction as opposed to the objective recording of survival or death.

The combining power tests show that the Lf dose has not altered upon activation, thus indicating that the total antigenic content remains unaltered. The L+ dose of this unactivated toxin is 1·9 times the Lf dose, indicating the presence of a considerable amount of proto-toxin with less affinity than toxin for antitoxin. After activation the L+ dose is within experimental error equal to the Lf dose. The change in volume of test doses after activation is an indication of the degree of conversion of prototoxin into toxin, and as these test doses can be determined with a high degree of accuracy, we are introducing the term "activation index" for the ratio of the L+, Ln or Lr doses before and after activation. Table I shows that the L+ and Ln doses of the unactivated toxin are not equal but of the same order of magnitude; after activation they were equal for this particular toxin, but the Lr in guinea-pigs was 1·44 times the Ln in rabbits. Considerable difficulty has often been encountered in fixing the Lr dose of unactivated toxin in guinea-pigs; certain factors are involved which have not yet been determined.

An analysis of the results of tests upon 16 short growth toxins before and after activation gave the following results:

1. The L+ dose in mice of unactivated toxins was usually within 20 per cent on either side of 1·6 times the Lf, but may vary from 1·33 to 2·05 times the Lf, indicating that the proportion of prototoxin already converted to toxin may differ considerably in filtrates from a 24-hour growth.

2. The L+ doses in mice of unactivated toxins were mostly about 20 per cent greater than the Ln in rabbits, but this may have been due to the quality of the standard antitoxin used. These two test doses are practically equal for activated toxins.

3. The Lr dose in guinea-pigs was about 1·5 times the Lf, L+ or Ln.

4. Activation ratios varied from approximately 20 to 150 or 200.

5. Activation indices measured by the change in L+ dose varied from 1·33 to 2·1.

6. As a general rule the L+ to Lf ratio of a toxin before activation (the activation index) varied with the magnitude of the activation ratio.

In filtrates from 4-day growths, prototoxin may be changed to toxin to such an extent that the activation ratio after treatment with trypsin is only from 2–5.

Accuracy of titrations.

It is our custom when testing the antitoxic value of any serum to include in the series of tests two sera that are used as substandards in order to determine whether the test toxin has deteriorated, to check the efficiency of the assistants doing the work, and to collect data in relation to the behaviour of two sera differing in quality. In this paper reference is made to three sera, A (RR.2364), a good avid, B (R.6615), slightly non-avid, and C (R.4182), a very non-avid antitoxin. The results of 29 titrations using 2 mice at each level are given in Table II, referring to intravenous injections into mice using an unactivated toxin ; Table III gives the results of 91 tests using an activated toxin. The results in Table II are obtained from the injection of a test dose of unactivated toxin equivalent to 10 units of antitoxin, and in Table III using an activated toxin with a test dose equivalent to 2·5 units of antitoxin. It can be seen that the neutralization curve is neither very steep nor very flat. Any series of results are affected by the accuracy of many measurements, by the character of the toxin and antitoxin under consideration, and by the conditions and degree of uniformity of the test animal. The true characteristic of a toxin in relation to an antitoxin can only be determined by special experiment with all factors carefully controlled. Curves plotted from Tables II and III would represent a characteristic involving not only the toxin and antitoxin concerned, but the general conditions of mice taken from large stocks supplied by different dealers, and also the accuracy and uniformity of a number of assistants making these routine tests. In the series of tests recorded one source of error was the use on several occasions of solutions of toxin several hours after they were made.

An analysis of the detailed results summarized in Table II has been used to

TABLE II.—*Showing the Degree of Accuracy of Titrations, by Intravenous Injection into Mice, of Unactivated* Cl. welchii *Epsilon Toxin* (*NX*.285) *and Antitoxin.*

Serum.	Test for (units per ml.).	Number of mice. Dying in (days)— 1	2	3	4	Living.	Percentage dying in— 1 day.	4 days.	Average death time in days.
A	165	4	0	2	1	51	7	12	2.00
	180	18	2	4	1	33	31	43	1·52
	200	45	7	1	2	3	78	95	1·27
B	200	1	0	5	0	52	2	10	2·67
	220	24	6	4	2	22	41	62	1·55
	240	39	6	8	0	5	67	91	1·42

assess the antitoxic titre of one substandard compared with the other ; if tests are made when the toxin solution has deteriorated, a comparison of values can still be made providing the deterioration is not beyond the range of tests. Such an analysis shows that out of 29 tests made at 10 per cent differences using 2 mice at each test, 2 gave no answer ; of the remaining 27, 16 (59 per cent) showed the antitoxic value of B to be 1·15 or 1·20 times that of A, and 24 (89 per cent) to be between 1·10 and 1·25, if the end point adopted was an equal number of survivals

and deaths at the end of 48 hours. If the end point was extended to 96 hours, 19 tests (70 per cent) showed B to be from 1·15 to 1·20 times A, and 24 (89 per cent) between 1·10 and 1·25. Similar figures for the activated toxin (Table III) were : No answer in 13 tests out of 90, in which both sera were used, and of the remaining 77, 43 (56 per cent) as 1·00 or 1·05, and 66 (86 per cent) between 0·95 and 1·10 if 48 hours was taken as the determining limit of time, and 45 (58 per cent) as 1·00 or 1·05, and again 72 (93 per cent) between 0·95 and 1·10 if the later death times were taken.

TABLE III.—*Showing the Degree of Accuracy of Titrations, by Intravenous Injection into Mice, of Activated* Cl. welchii *Epsilon Toxin* (*NX*.316) *and Antitoxin.*

Serum.	Test for (units per ml.).	Number of mice. Dying in (days)— 1	2	3	4	Living.	Percentage dying in— 1 day.	4 days.	Average death time in days.
A	165	16	2	1	0	163	9	10	1·21
	180	51	5	7	4	115	28	37	1·46
	200	140	6	5	1	28	78	85	1·12
	220	165	4	2	2	9	91	95	1·09
B	165	15	1	2	0	164	8	10	1·28
	180	38	7	4	2	131	21	28	1·41
	200	111	5	10	9	45	61	75	1·38
	220	165	1	4	1	11	91	94	1·06

In our experience careful selection of mice reduces the number of deaths occurring out of order, use of freshly dissolved toxin avoids the occasional unsatisfactory test, and titrations by expert assistants bring this type of test to a high degree of accuracy.

Tables IV and V give similar results for control tests for intracutaneous injection into rabbits and Table VI into guinea-pigs, using the same substandard sera. The symbols used for recording reactions are — for no reaction, S for a small indefinite reaction which might be non-specific or might be a very small definite reaction, ± for a border line but definite reaction, and + for any fully developed reaction. Tests are made in rabbits on both types of toxin at a 1 unit level and in guinea-pigs at a level of 0·2 unit.

TABLE IV.—*Showing the Degree of Accuracy of Titrations, by Intracutaneous Injection into Rabbits, of Unactivated* Cl. welchii *Epsilon Toxin* (*NX*.285) *and Antitoxin.*

Serum.	Test for (units per ml.).	Number of tests showing reactions recorded as— —	S.	±	+	Percentage number of positive reactions.
B	200	32	5	4	1	12
	220	7	8	15	12	64
	240	2	3	2	35	88
	270	0	0	2	40	100

— = No reaction. S. = Small indefinite reaction. ± = Border line but definite reaction. + = Fully developed reaction.

TABLE V.—*Showing the Degree of Accuracy of Titrations, by Intracutaneous Injection into Rabbits, of Activated* Cl. welchii *Epsilon Toxin* (*NX*.316) *and Antitoxin.*

Serum.	Test for (units per ml.).	Number of tests showing reactions recorded as—				Percentage number of positive reactions.
		—	S.	±	+	
A	165	18	11	3	4	19
	180	13	6	6	11	47
	200	4	2	1	29	83
	220	1	1	1	33	94
B	180	35	1	0	0	0
	200	22	6	4	4	22
	220	4	5	11	16	75
	240	1	2	1	32	93

TABLE VI.—*Showing the Degree of Accuracy of Titrations, by Intracutaneous Injection into Guinea-pigs, of Activated* Cl. welchii *Epsilon Toxin* (*NX*.316) *and Antitoxin.*

Serum.	Test for (units per ml.).	Number of tests showing reactions recorded as—				Percentage number of positive reactions.
		—	S.	±	+	
A	165	37	7	3	5	15
	180	17	15	6	14	38
	200	7	9	8	28	69
	220	2	2	2	46	92
B	180	41	7	1	3	8
	200	24	9	3	16	36
	220	9	6	10	27	71
	240	6	6	5	35	77

These results show that the accuracy of tests using the intracutaneous method in rabbits or guinea-pigs is of the same order as that given by intravenous injection into mice. Table VII records the antitoxic values of the two sera determined

TABLE VII.—*Showing Calculated Antitoxic Value in Units per ml. of Two Standard Sera Tested by Different Methods.*

Method of injection.	Intravenous injection.		Intracutaneous injection.		
Animal.	Mouse.		Rabbit.		Guinea-pig.
Toxin.	Unactivated.	Activated.	Unactivated.	Activated.	Activated.
Serum A	183	185	..	182	188
Serum B	215	189	214	211	216
Number of observations.	58	182	42	36	52

by calculating the amount of each serum which mixed with a test dose of toxin would produce after injection an equal number of survivals and deaths or reactions and no reactions according to the method of testing. The number of mice used at each level tested is sufficient to show that the value of B in relation to A is significantly different according to whether activated or unactivated toxin is

used in the mouse intravenous method. This difference is due to a difference in avidity of the two antitoxic sera. The uniformity of all other values is a confirmation of the accuracy of determination of the test doses of the toxins.

Avidity.

Antitoxic sera differ in relation to the degree of firmness with which they combine with toxin. Non-avid antitoxin combines loosely with toxin and mixtures dissociate upon dilution. Mixtures of toxin and non-avid antitoxin which are over-neutralized when judged by the flocculation test are toxic to animals, and the *in vivo* value assigned to a non-avid antitoxic serum is less than the *in vitro* value. Mixtures may continue to dissociate after injection and toxin is slowly freed, causing late deaths. The difference in the firmness of combination between antitoxin and toxin and that of antitoxin and toxoid is far greater with a non-avid antitoxin; this explains the decreased efficiency of the serum B when tested in mice against an activated toxin. Glenny, Pope and Waddington (1925) showed that mixtures of diphtheria toxin with non-avid antitoxin could be prepared in such proportions that the intracutaneous injection of 0·2 ml. into guinea-pigs causes no reaction, while 0·2 ml. of a 1 in 10 dilution of the mixture would produce large reactions. Glenny and Barr (1932), also referring to diphtheria toxin and antitoxin, stated that "the simplest measure of firmness of combination is the dilution ratio, which is the amount of antitoxin necessary to neutralize the Lr dose of toxin in a total volume of 2 ml., divided by the amount required to neutralize the same dose in 200 ml."

No attempt has yet been made by us to fix the dilution to be used to determine any definite dilution ratio as a measure of avidity of epsilon antitoxin, but a few comparisons have been made of titrations of certain sera at different levels. Table VIII shows a comparison made between titrations of sera A and B at three

TABLE VIII.—*Showing the Effect of Dilution upon Mixtures of Antitoxin and an Activated Toxin (NX.316).*

Serum.	Test for (units per ml.).	Number of deaths in mice out of total injected intravenously with 0·5 ml. of mixture diluted to—		
		2 ml.	10 ml.	100 ml.
A	150	2/22	1/22	..
	165	16/28	11/28	0/21
	180	18/24	18/24	2/21
	200	14/14	16/18	10/26
	220	..	..	13/18
	240	..	..	10/10
B	135	..	0/6	0/8
	150	..	0/4	3/4
	165	1/10	3/10	6/6
	180	2/6	4/6	8/8
	200	7/10	8/8	..
	220	4/4	..	..
		Calculated value.		
A	..	163	170	207
B	..	189	173	160

different levels. Mixtures of 40 L+ doses and appropriate dilutions of serum were made in volumes of 2, 10 and 100 ml., and 0·5 ml. of the diluted mixtures were injected intravenously into mice. More mice died after injection of dilutions of mixtures of toxin and antitoxin B than died after injection of less dilute mixtures, showing that B was not avid. On the other hand, dilutions of mixtures containing A caused fewer deaths than less dilute mixtures because combination between toxin and an avid antitoxin was relatively firm, and dilutions reduced the available free toxin. Thus the relative value of a non-avid antitoxin to an avid one depends upon the amount of toxin used in the test dose.

If A were regarded as a fixed standard with an antitoxic content of 185 units per ml. (Table VII) and test doses were adjusted at each level of testing, then the apparent titre of B would be 215, 189 or 132 units per ml. according to the level at which tests were made. These figures emphasize the point that *in vivo* titrations cannot give an absolute value to a non-avid antitoxin. The flocculation test alone shows the total antitoxic content ; *in vivo* tests show how much antitoxin is available to bind toxin under the particular conditions of the experiment.

Table IX gives the results of titrations at two levels of C, a less avid antitoxin

TABLE IX.—*Showing the Effect of Dilution upon Mixtures of Antitoxin and an Activated Toxin* (*NN*.1314).

Serum.	Test for (units per ml.).	Number of deaths in mice out of total injected intravenously with 0·5 ml. of mixture diluted to :—	
		2 ml.	20 ml.
B	200	..	2/6
	220	0/6	4/6
	240	1/6	6/6
	270	6/6	..
C	220	..	0/9
	240	..	2/9
	270	..	8/9
	300	..	8/9
	330	0/9	9/9
	360	3/9	..
	400	6/9	..
		Calculated value.	
B	..	252	210
C	..	380	257

compared with B ; at the strongest level of testing C appears to contain 380 units per ml., but only 257 if one-tenth of the amount of toxin is used for titration. Thus the two Tables VIII and IX show that A is avid, B is less avid, and C still less avid ; that the avid serum appears more effective when less toxin is used unless the fractional test doses are adjusted, but the non-avid sera are less effective against smaller doses of toxin ; consequently the values of non-avid antitoxins in relation to avid ones appear lower if less toxin is used as the basis of comparison. The L+ doses of the toxins used in the experiment recorded in Table IX have not been correctly adjusted ; the object of the experiment was to compare apparent values at different dilutions rather than to determine exact values.

In titrations of avid antitoxin against activated toxin by the mouse intravenous method, a certain number of mice die after the first 24 hours, and less after 48 hours, but the number so dying increases when titrations are made against unactivated toxin, and still further increases when non-avid antitoxin is titrated. Results given in Table III (activated toxin) show that of the mice used for testing serum B, 8·1 per cent of all deaths occurred after 48 hours and in Table II (unactivated toxin) 20·0 per cent; the corresponding figures for the avid serum A were 5·3 per cent and 12·8 per cent. Many more late deaths occurred when the least avid C was tested. Results are given in Tables X and XI, showing that the percentage of late deaths occurring among mice used for testing C reached 89 per cent, compared with 44 per cent with B when an unactivated toxin was used and 30 per cent compared with 9 per cent using an activated toxin.

No single set of figures can express differences in titration curves of one serum compared with another against the same toxin, or of one serum against different toxins; account must be taken of the number of mice dying and of the time of death. It is of advantage to adopt some standard method of expressing a combination of the number of animals dying and the average death time. We suggest the term "lethal index" for the product of the percentage number of animals dying and a factor representing the acceleration in death time. Since in our experiments mice were not observed after the fourth day, those dead on that day died at least one day earlier than those living on that day, so we assume the acceleration in death time to be one day, and for those dying on the first day to be 4 days. Thus factors of 1·00, 0·75, 0·5, 0·25 are allotted for average death times of 1, 2, 3 and 4 days respectively. The formula for the lethal index thus becomes the percentage number dying multiplied by the average death time in days subtracted from 5 and divided by 4. For example the lethal index for the titration in Table X of serum B for 200 units per ml. becomes—

$$\frac{9 \times 100}{\cdot 48} \times \frac{5 - 2\cdot 78}{4} = 10\cdot 4$$

It must be pointed out that a lethal index of this type is only applicable if cause and effect are in a linear relationship. As late deaths are due to slow dissociation this method would appear applicable.

TABLE X.—*Comparing the Number of Late Deaths among Mice Injected Intravenously with Mixtures of an Unactivated Toxin (NX.285) with Varying Amounts of Two Sera of Different Degrees of Non-avidity.*

Serum.	Test for (units per ml.).	Number of mice. Dying in (days)— 1	2	3	4	Living.	Percentage dying in— 1 day.	2 to 4 days.	Average death time in days.
B	200	3	0	2	4	39	6	12	2·8
	220	7	7	6	4	24	15	35	2·3
	240	24	15	6	0	3	50	44	3·6
	270	39	3	0	0	0	93	7	3·1
C	300	0	0	4	7	31	0	26	3·6
	330	0	2	19	5	18	0	59	3·1
	360	1	13	22	6	6	2	85	2·8
	400	3	24	15	4	2	7	89	2·3
	450	7	9	0	0	0	44	56	1·6

Results from Table X can now be compared graphically in Fig. 1, the darkened areas representing the percentage number of mice dead on or before the fourth day; the black area represents the lethal index and the shaded area varies in length with the number and time of late deaths. The absence of any shaded area, as in the fourth column in Fig. 2, shows that all mice died on the first day. The shaded areas in Fig. 1 show that many more late deaths occurred in titrations of C than of B; it can also be seen that the neutralization curve for the very non-avid antitoxin is less steep than that of the slightly non-avid if judged by the proportion of mice dying, and still less steep if judged by the lethal index. Results of the same two sera against an activated toxin (Table XI) shown in Fig. 2 afford a contrast with those shown in Fig. 1.

TABLE XI.—*Comparing the Number of Late Deaths among Mice Injected Intravenously with Mixtures of an Activated Toxin (NX.308) with Varying Amounts of Two Sera of Different Degrees of Non-avidity.*

Serum.	Test for (units per ml.).	Number of mice.					Percentage dying in—		Average death time in days.
		Dying in (days)—				Living.	1 day.	2 to 4 days.	
		1	2	3	4				
B	165	4	0	0	0	18	18	0	1·0
	180	7	1	1	0	13	32	9	1·3
	200	20	0	2	0	0	91	9	1·2
	220	22	0	0	0	0	100	0	1·0
C	270	2	0	0	0	14	12	0	1·0
	300	9	4	2	0	5	41	30	1·53
	330	13	5	0	0	2	59	25	1·28
	360	6	2	0	0	0	75	25	1·2
	400	4	0	0	0	0	100	0	1·0

Fig. 3, prepared from figures given in Table II, can be compared directly with Fig. 1, as titrations were made against the same unactivated toxin. There is very little difference between the diagram for the avid antitoxin A and that for the slightly non-avid one B. The difference in avidity of these two sera is considerable when judged by the effect of dilution upon toxin antitoxin mixtures (Table VIII). It would appear therefore that many late deaths and flat neutralization curves are properties only of very non-avid antitoxin such as C.

The three sera were tested against a short growth toxin before and after activation; tests were made at the same time under comparable conditions, using 10 mice at each level. As no early deaths followed the injection of the first series of tests up to 360 units on serum C against unactivated toxin, a further series of 10 mice were used on tests from 360 to 550 units. Results are given in Table XII. All tests were made against a test dose equivalent to 10 units. Only 1 mouse out of 40 died after the 2nd day when injected with mixtures of the avid serum A with unactivated toxin, 10 out of 40 injected with mixtures containing B, and 38 out of 90 when C was used. All 20 mice at the 360 unit level died on the 2nd, 3rd or 4th day. In all the tests using the same toxin after activation only 3 mice out of 110 died after the 2nd day and only 4 on that day.

Figs. 4 and 5 show graphically the results given in Table XII. In the diagram for C in Fig. 4 one area for the 360 test is shaded by diagonal lines in two directions

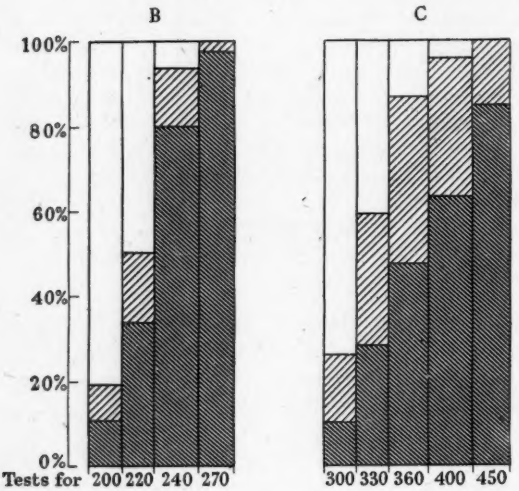

FIG. 1.—Comparing the results of titrations, by intravenous injection into mice, of slightly non-avid serum B with those of the least avid serum C, using an unactivated toxin NX.285 (Table X).

The height of the darkened columns gives the percentage number of mice dying, that of the shaded columns indicates the delay in death time and that of the black column the acceleration in death time between 1 and 5 days.

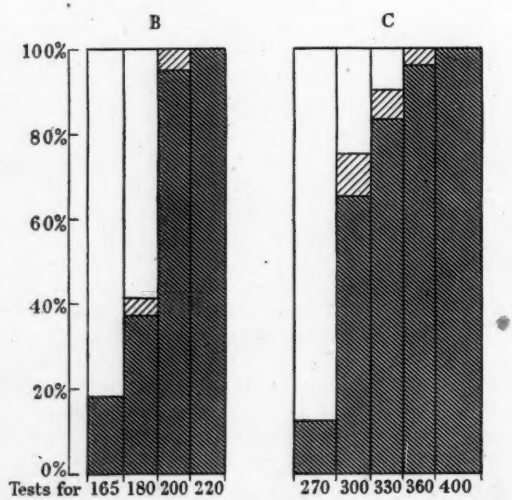

FIG. 2.—Comparing the results of titrations, by intravenous injection into mice, of slightly non-avid serum B with those of the least avid serum C using an activated toxin NX.308 (Table XI).

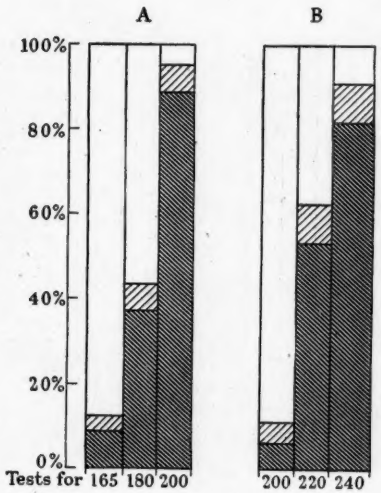

FIG. 3.—Comparing the results of titrations, by intravenous injection into mice, of the avid serum A with those of the slightly non-avid B using an unactivated toxin NX.285 (Table II).

TABLE XII.—*Showing the Titration by Intravenous Injection into Mice of 3 Sera against the Same Toxin (NX.327) Before and After Activation.*

Anti-toxin.	Test for (units per ml.).	Unactivated toxin. Number of mice dying in (days)— 1	2	3	4	Living.	Average death time in days.	Activated toxin. Number of mice dying in (days)— 1	2	3	4	Living.	Average death time in days.
A	165	0	4	0	0	6	2·0	—	—	—	—	—	..
	180	2	3	0	1	4	2·0	0	0	0	0	10	..
	200	5	5	0	0	0	1·5	7	0	1	0	2	1·25
	220	9	1	0	0	0	1·1	10	0	0	0	0	1·00
B	180	0	0	0	0	10	..	0	0	0	0	10	..
	200	0	0	1	3	6	3·8	3	0	1	0	6	1·50
	220	3	2	2	2	1	2·3	10	0	0	0	0	1·00
	240	5	2	1	1	1	1·8	—	—	—	—	—	..
C	270	0	0	1	4	5	3·8	0	0	0	0	10	..
	300	0	0	4	2	4	3·3	1	0	1	0	8	2·0
	330	0	2	2	3	3	3·1	7	3	0	0	0	1·3
	360	0	6	9	5	0	2·9	9	1	0	0	0	1·1
	400	1	2	7	0	0	2·6	10	0	0	0	0	1·0
	450	2	7	1	0	0	1·9	—	—	—	—	—	..
	500	7	2	1	0	0	1·4	—	—	—	—	—	..
	550	9	1	0	0	0	1·1	—	—	—	—	—	..

to indicate the difference between the results on the two occasions when this test was made. The upper limit corresponds to the blackened area for the first series of tests and the lower limit for the second series. The difference in the two sets of results amounted to 0·5 day in the average death time. This can be partially explained by the fact that the mice in the second series were injected 6 hours later in the day than those in the first series, but readings of deaths were taken at the same time on both series, so that a death on the second day in the first series was a death within 46 hours, and in the second series within 40 hours. The

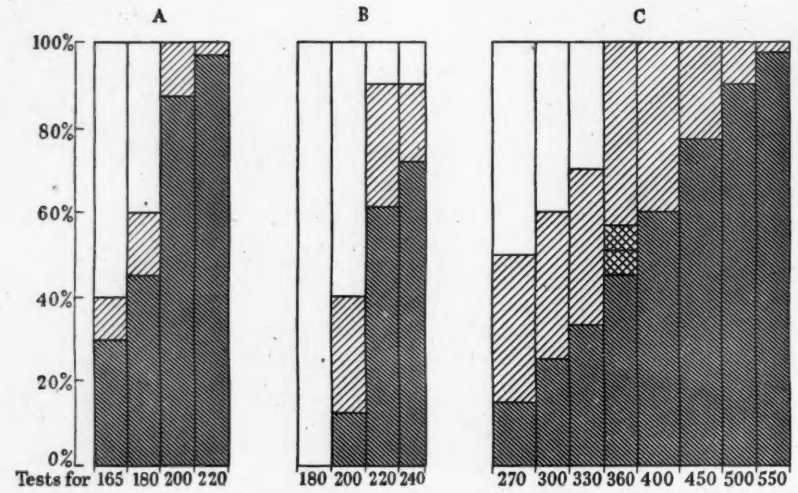

FIG. 4.—Comparing the results of titrations, by intravenous injection into mice, of 3 sera, A, B and C, using an unactivated toxin NX.327 (Table XII).

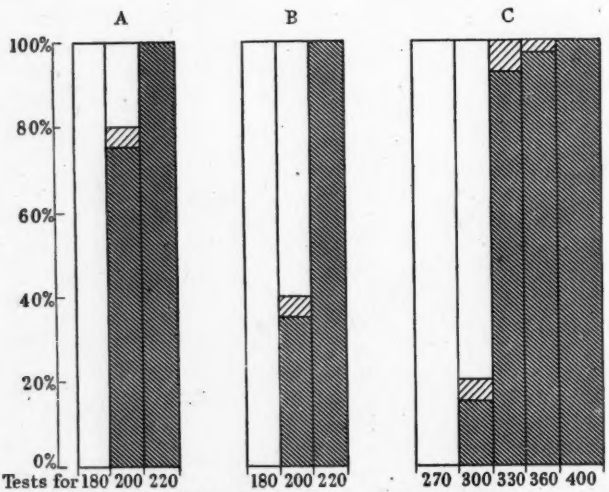

FIG. 5.—Comparing the results of titration, by intravenous injection into mice, of 3 sera, A, B and C, using toxin NX.327 after activation (Table XII).

slope therefore of the curve for lethal index would not be quite so flat as in the diagram.

The results recorded in various tables are summarized in Tables XIII and XIV, showing the ratios of the apparent antitoxic titres of the three sera determined by intravenous injection into mice. More late deaths occur in titrations

TABLE XIII.—*Showing the Comparative Antitoxic Strength of Three Sera Determined by Intravenous Injection into Mice against Activated and Unactivated Toxins, judging the End Point of Titration by 50 per cent Survivals at end of* (a) *1st day*, (b) *2nd day*, (c) *4th day*.

Table.	Toxin No.	Type of toxin.	Amount of toxin used (L+ doses).	Ratio of antitoxic titre. $\frac{B}{A}$ (a)	(b)	(c)	$\frac{C}{A}$ (a)	(b)	(c)	$\frac{C}{B}$ (a)	(b)	(c)
III	NX.285	Unactivated	10·0	1·21	1·19	1·19	..	..	..	..	..	..
X	NX.285	„	10·0	..	..	..	..	..	..	>1·88	1·71	1·42
XII	NX.327A	„	2·5	1·20	1·22	1·18	2·40	2·32	1·56	2·00	1·90	1·32
II	NX.316	Activated	2·5	1·03	1·03	1·02	..	..	..	..	..	..
XI	NX.308	„	2·5	..	..	..	..	..	..	1·65	1·57	1·57
XII	NX.327B	„	2·5	1·06	1·06	1·05	1·65	1·61	1·61	1·55	1·52	1·53

TABLE XIV.—*Showing the Comparative Antitoxic Strength of Three Sera Determined by Intravenous Injection into Mice against Multiples and Submultiples of Usual Test Doses of Activated Toxins, judging the End Point of Titration by 50 per cent Survival at end of 4 days.*

Table.	Toxin No.	Amount of toxin injected (L+ dose).	Ratio of antitoxic titre $\frac{B}{A}$	Table.	Toxin No.	Amount of toxin injected (L + dose).	Ratio of antitoxic titre $\frac{C}{B}$
VIII	NX.316	10·0	1·16	11	NX.308	2·5	1·57
XII	NX.327B	2·5	1·05	12	NX327.B	2·5	1·53
II	NX.316	2·5	1·02	9	NN.1314	2·5	1·52
VIII	NX.316	2·0	1·02	9	NN.1314	0·25	1·25
VIII	NX.316	0·2	0·77				

with non-avid than with avid antitoxin; it therefore follows that the relative titres appear to alter according to whether the end point chosen is 50 per cent survival after 24, 48 or 96 hours. Relative values are also affected by the type of toxin used for titrations and the level at which titrations are made relative to the L+ dose.

Of the two unactivated toxins NX.285 was a solution of toxin precipitated by ammonium sulphate and dried. In our experience this process frequently reduces the number of lethal doses in the L+ dose; NX.285, although prepared from a long growth filtrate, only contained 7 mouse lethal doses in the test dose equivalent to 10 units; NX.327A had not been precipitated or dried, and was a filtrate from a 24 hours' growth containing 20 lethal doses in the test dose equivalent to 2·5 units.

Several factors contribute to the size of the ratios of the antitoxic values of the three sera calculated from the results of the various tests. The day on which readings are made to determine 50 per cent survivals has practically no effect upon the relative values of A and B, and there is little difference between the apparent strength of the least avid serum C in relation to the other two, whether readings be made on the first or on the second day, but the ratio of the value of the serum to either A or B falls considerably (from 2·4 to 1·56 or from 2·0 to 1·32) if readings are postponed until the fourth day, when titrations are made with unactivated toxin, but not when activated toxin is used.

The antitoxic values of non-avid sera appear relatively higher when unactivated toxin is used than when the test toxin is activated. The slightly non-avid B

compared with the avid A appears about 15 per cent higher when titrated against the unactivated as compared with the activated toxin. The least avid serum C in relation to A appears 50 per cent higher against the unactivated toxin if end points of death or survival are taken after one or two days, but approximately the same if the readings are made on the fourth day, although the less non-avid B appears about 12 per cent higher with the non-activated toxin when judged by this end point. It follows that the comparison between the least avid C and the less avid B shows a higher ratio against unactivated than against activated toxin, judged by readings after 1 or 2 days and the reverse at the end of 4 days. The concentration of mixtures injected (Table XIV) has some effect (about 10 per cent) on the relative titres of non-avid antitoxin titrated against activated toxin in the range from 10 L+ doses of toxin in the mixture to 2 or 2·5 L+ doses, but a considerably greater effect when the amount of toxin is reduced to 0·2 or 0·25 L+ doses.

Most of the tests recorded have been made upon routine toxins prepared by Mr. J. Macsween, and we are specially indebted to him for his co-operation in preparing some special batches of toxin for experimental purposes.

SUMMARY.

1. The flocculation reaction can be used to determine the total antigen content of *Cl. welchii* epsilon toxins both before and after activation by trypsin provided the antitoxin used in the test has been refined by the pepsin process.

2. Antitoxic values of sera can be estimated by the flocculation reaction if the test toxin has been activated by trypsin.

3. Toxicity of filtrates can be determined by intravenous injection into mice and by intracutaneous injection into rabbits and guinea-pigs.

4. Combining power tests can be made by the same methods with one reservation, namely that only trypsin activated filtrates are suitable as test toxins for the titration of antitoxin in the skin of guinea-pigs.

5. The combining power of toxins or the antitoxic value of sera can be measured with a high degree of accuracy by intravenous injection into mice.

6. Titration of non-avid antitoxin by *in vivo* methods gives results that vary considerably with conditions of test.

7. Mice injected intravenously with mixtures of activated toxin and avid antitoxin usually survive for many days if they do not die within 24 hours ; if the mixtures contain unactivated toxin and non-avid antitoxin many mice surviving 24 hours die during the following days.

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BODILY REACTIONS TO TRAUMA. THE INFLUENCE OF ISCHAEMIA ON THE CLOTTING FACTOR OF MUSCLE.

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IT is generally held that tissue damage leads to the liberation of a clotting factor—thromboplastin. Evidence for this view appears to have been derived, in the main, from indirect observations, and although the chemical nature and properties of thromboplastin have been intensively investigated in recent years, little attention has been paid to its function under physiological and pathological conditions. The experiments of Quastel and Racker (1941) are therefore of special interest, since they showed that anoxaemia of tissues (brain, liver, kidney and striated muscle) increased the clot-promoting activity of extracts prepared from them. They thought that this might have a bearing on the problem of the general bodily reaction to tissue trauma. This idea is supported by the results of Konig (1932) and Dyckerhoff and Goevenich (1939), who investigated the toxicity of muscle extracts and concluded that it was due to two components, myotoxin and thromboplastin. Myotoxin has been identified as adenosine triphosphate by Bielschowsky and Green (1943), and we have recently shown (Green and Stoner, 1947) that the intravenous toxicity of fresh muscle and other tissue extracts can be explained by their nucleotide and thromboplastin contents. The findings of Quastel and Racker (1941) therefore gain importance, and the results communicated here form an amplification of their experiments.

METHODS.

Muscle specimens were obtained from the hind limbs of albino rats. Ischaemia was produced by the metal clamp usually used in this department (Green, 1943) applied to one limb under ether narcosis. Although this clamp produces a severe degree of ischaemia, it does not completely occlude the blood supply to the limb. Except where otherwise stated the period of ischaemia was 5 hours since, under our experimental conditions, clamping of both hind limbs for this period is fatal. The rats were killed by a blow on the head followed by bleeding from the neck. The clamp was then removed and the hind limb muscles sampled. In some experiments sampling was preceded by perfusion of the hind limbs with 0·9 per cent NaCl injected into the abdominal aorta just above its bifurcation. For some experiments the muscle from the normal and ischaemic limbs of two rats was pooled to give sufficient volume of extracts. The muscle samples were rapidly weighed and ground up with quartz and twice their volume of 0·9 per cent NaCl. Extraction was allowed to continue for $\frac{1}{4}$ hour, when the mixture was filtered on a Buchner funnel (Whatman No. 1 paper). This filtrate was refiltered by gravity through Whatman No. 1 paper and the resultant turbid extract stored in the refrigerator.

The effect of these extracts on the clotting of freshly prepared oxalate rabbit plasma (0·5 ml. 0·1 M sodium oxalate to 4·5 ml. blood) was determined on the following day. Rat and human plasma were also used in a few experiments. The clotting time (C.T.) of the recalcified plasma was determined in uniform glass tubes (74 × 9 mm.) according to the method of Quick (1942). 0·1 ml. of extract was placed in the tube with 0·02 M $CaCl_2$ and 0·1 ml. of plasma added. The tube was then agitated in a water bath at 37° C., and the time from the addition of the plasma to the first appearance of fibrin threads was determined with a stop-watch. The control C.T. of the plasma was determined by replacing the extract by an equal volume of 0·9 per cent. NaCl in the reaction mixture.

In a few experiments fibrinogen was used instead of oxalate plasma. The fibrinogen was either a pure preparation of human fibrinogen* or a preparation made from rabbit plasma by Mellanby's method (1908). This latter fibrinogen is not free from prothrombin. Prothrombin-poor plasma was also substituted for oxalate plasma in some of the experiments being prepared by treating the oxalate plasma with aluminium hydroxide (Quick, 1935).

Control specimens of thromboplastin were prepared from rat brain the day before use by acetone drying, followed by storage in a vacuum desiccator and extraction with 0·9 per cent NaCl before use (Quick, 1942). For some tests similar preparations were made from muscle samples. For the extraction of the acetone dried muscle samples a volume of 0·9 per cent. NaCl equal to twice the volume of the original muscle samples was used.

In experiments on fibrinolysis the technique of Macfarlane and Pilling (1946) was followed. The effect of the extracts on clots formed in 1 : 15 and 1 : 30 dilutions of human and rat plasma was observed and also on clots formed in 0·25–1·4 per cent solutions of human fibrinogen by the addition of thrombin (Parke Davis).

The following chemical methods were used: In testing extracts for tryptic activity their effect on a 10 per cent casein solution was followed by formol titration (Cole, 1933). To determine the total phosphorus and iron contents of the extracts portions were first ashed with a mixture of equal volumes of concentrated sulphuric and nitric acids. The total phosphorus was then determined by Briggs' method (Peters and Van Slyke, 1932), and the total iron by its reaction with thioglycolic acid (Tompsett, 1934). The phospholipin content of the muscle was determined on samples which were first minced and dried with anhydrous sodium sulphate. The dried muscle was then extracted for 6 hours in a soxhlet extractor with a 1 : 1 alcohol-ether mixture (Cohen and Chargaff, 1940). The solvents were removed by vacuum distillation and an ether extract of the residue made. The phospholipins were precipitated from this by acetone and alcoholic magnesium chloride (Bloor, 1943), and the phosphorus content of the precipitate was determined, after ashing, as above. The phospholipin content of the saline extracts was similarly determined after the phospholipins had been extracted by adding a portion of the extract to 25 volumes of a 3 : 1 alcohol-ether mixture and allowing it to stand for 1 hour at room temperature. The total protein N of the extracts was determined from the difference between the total N (Kjeldahl) and the N.P.N. (Folin-Wu) contents.

Throughout these experiments glass distilled water was used in the preparation of the solutions.

* We are indebted to the Lister Institute for this sample

RESULTS.

Effect of extracts of normal and ischaemic muscle on the clotting time of recalcified oxalate plasma.

In agreement with Quastel and Racker (1941), it was found that whilst normal muscle extracts accelerated the clotting of recalcified oxalate plasma (rabbit, rat, human), ischaemic muscle extracts were much more active (Table I). In this table, as in the others, the figures given are a representative selection of the total experiments. Since the C.T. under these conditions frequently approached the " prothrombin time " of the plasma the difference between the two extracts was often more obvious on dilution (Fig. 1). Very little difference was found in the activity of extracts prepared from the pooled normal muscles of the right and left hind limbs of two rats (Fig. 2).

TABLE I.—*The Effect of Saline Extracts of Normal and Ischaemic Rat Muscle on the Coagulation of Oxalate Rabbit Plasma at 37° C. Reaction Mixture contained 0·1 ml. 0·02 M $CaCl_2$ or 0·9 per cent NaCl 0·1 ml. Extract or 0·9 per cent NaCl and 0·1 ml. Plasma.*

Rat No.	Extract.	Coagulation time.	
		Recalcified oxalate plasma.	Oxalate plasma.
11–12*	Saline control	2′ 40″	∝
	Normal	44″	∝
	Ischaemic	21″	4′ 49·5″
13–14*	Normal	33·5″	∝
	Ischaemic	20·5″	33′ 14·5″
15–16*	Saline control	4′ 10″	∝
	Normal	25″	∝
	Ischaemic	20″	∝
17–18	Normal	26·5″	∝
	Ischaemic	17·5″	∝
19–20	Saline control	4′ 9″	∝
	Normal	45″	∝
	Ischaemic	22″	∝
21–22	Normal	35·5″	∝
	Ischaemic	20″	13′ 43″
24–25	Saline control	2′ 40″	∝
	Normal	37″	∝
	Ischaemic	22″	∝
26–27	Normal	57″	∝
	Ischaemic	20″	∝
65	Saline control	3′ 25″	
	Normal	23″	
	Ischaemic	15″	
66–67	Normal	22·5″	
	Ischaemic	18″	

* = Perfusion of hind quarters with 0·9 per cent NaCl before removal of muscles.

To determine the period of ischaemia necessary for the production of the maximal effect a method had to be used in which the activity of different extracts

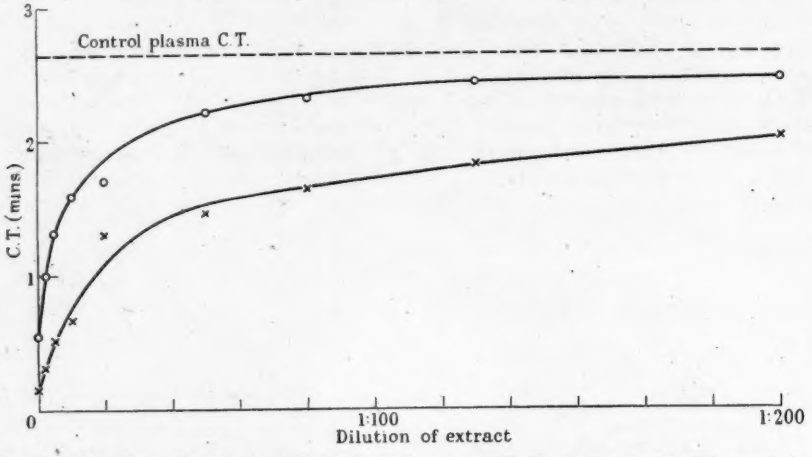

FIG. 1.—The effect of increasing dilutions of 0·9 per cent NaCl extract (1 : 2) of the pooled muscle from the normal (○—○) and ischaemic (×—×) limbs of two rats on the coagulation of recalcified oxalate rabbit plasma. Period of ischaemia = 5 hours.

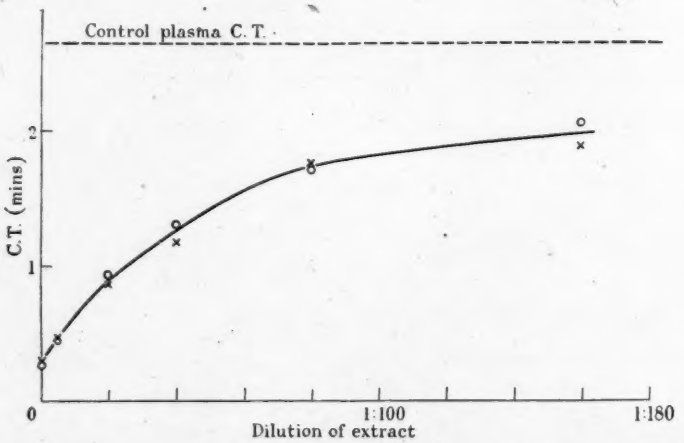

FIG. 2.—The effect of increasing dilutions of 0·9 per cent NaCl extracts (1 : 2) of the pooled muscle from the normal hind limbs of two rats on the coagulation of recalcified rabbit plasma.

○ = extract from right hind limbs.
× = „ left „

tested on different specimens of plasma could be compared. For this the formula proposed by Fischer (1935), $\frac{1}{t} = k.c^n$, where t is the C.T. in secs., c the concen-

tration of thromboplastic substance and k and a are constants, was used in its logarithmic form:

$$\log t = -a \log c - b \text{ where } b = \log k,$$

since Barratt (1934) and Astrup and Darling (1942) have shown that it can be applied to mammalian plasma.

The constants a and b were determined for our experimental conditions as described by Astrup and Darling (1942) and were found to be 0·354 and −1·7782 respectively. The concentration of thromboplastic substance could then be derived, after determining the C.T. in seconds, from a curve constructed to the formula—

$$\log c = \frac{1{\cdot}7782 - \log t}{0{\cdot}354}$$

The values obtained for c represent the concentration of thromboplastic substance in units where 1 unit is the amount required to clot 0·1 ml. plasma in 60 sec. There are objections to the use of the Fischer formula in this connection, but at present it appears to be the only practical solution to this problem (Astrup, 1944).

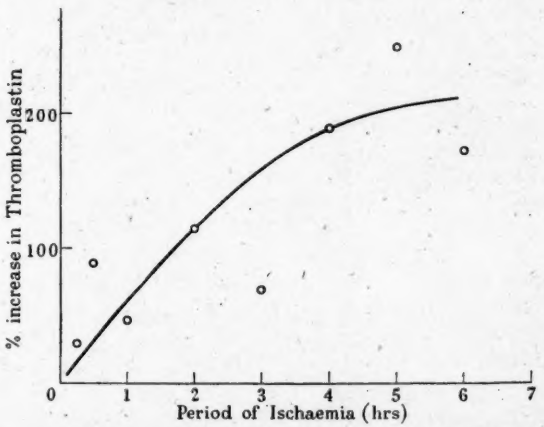

FIG. 3.—Shows the percentage increase in the thromboplastin content of the 0·9 per cent NaCl extract (1 : 2) from the ischaemic muscle of the rat's hind limb after varying periods of ischaemia. Each point represents the average of observations on three rats.

When extracts of the hind limb muscles after different periods of ischaemia were thus compared with extracts from the normal muscle of the opposite limb, it was found that the thromboplastic activity of the ischaemic muscle extract rose rapidly with increasing periods of ischaemia (Fig. 3). The maximum increase occurred after 4–6 hours' ischaemia, when the activity of the ischaemic muscle extract was 200 per cent greater than that of the normal muscle extract. The average difference in the activity of extracts prepared from each hind limb of a normal untreated rat was 35 per cent. With periods of ischaemia longer than 6 hours the greater activity of the ischaemic muscle extract was maintained.

After 17 hours ischaemia (the time used by Quastel and Racker and the longest period used in our experiments), the activity of the ischaemic muscle extract averaged 250 per cent more than that of the normal muscle extract.

This increase in activity after muscle ischaemia was not seen in muscle specimens which had been allowed to autolyse either aerobically at room temperature or at 37° C. or anaerobically at 37° C.

Properties of the clotting factor in normal muscle extracts.

In the preceding paragraphs it has been assumed that the clot-promoting activity of these muscle extracts is due to thromboplastin. This was first suggested by Quastel and Racker (1941), and our results fully confirm this. Ca^{++} is necessary for the action of these extracts (Table I), and they will not clot pure human fibrinogen. The thromboplastic nature of the compound is also suggested by the longer C.T. when prothrombin-poor plasma and Mellanby-fibrinogen were used (Table II). These experiments establish that the muscle-clotting factor acts in the first equation of the classical scheme for blood coagulation.

TABLE II.—*The Effect of Saline Extracts of Normal and Ischaemic Rat Muscle on the Coagulation of Recalcified Oxalate Plasma, "Prothrombin-Poor" Plasma and Mellanby-Fibrinogen.*

Rat No.	Extract.	Coagulation time. Recalcified oxalate plasma.	Recalcified "prothrombin-poor" plasma.	Recalcified Mellanby-fibrinogen.
	Saline control	4′ 10″		
15–16	Normal	25″	3′ 35″	
	Ischaemic	20″	3′ 35″	
17–18	Normal	26·5″	4′ 2″	
	Ischaemic	17·5″	3′ 10″	
	Saline control	2′ 48″	Slight clot after 30′	
103	Normal	24″		2′ 58″
	Ischaemic	18″		1′ 42″
104	Normal	16·5″		2′ 48″
	Ischaemic	15″		2′ 48″
105	Normal	23″		3′ 5″
	Ischaemic	15″		2′ 27″

TABLE III.—*The Effect of Centrifugation and Seitz Filtration on the Activity of Saline Muscle Extract and Saline Extract of Acetone Dried Muscle.*

Treatment.	C.T. of recalcified oxalate plasma. 0·9 per cent NaCl extract.	0·9 per cent NaCl extract of acetone dried muscle.
Nil	27″	1′ 2″
After centrifugation for ½ hour at 2000 r.p.m.	30·5″	1′ 55″
After Seitz filtration	4′ 4″	3′ 0″
Saline control	3′ 42″	3′ 7″

It is of interest to see how closely this muscle thromboplastin resembles those obtained from other tissues such as lung and brain. The studies of Mills, Chargaff and Quick have shown that thromboplastin from these sources contains protein and phospholipin, both of which are necessary for its activity and its chemical behaviour is a function of both groups.

The activity of a normal muscle extract was reduced by centrifugation and destroyed by Seitz filtration (Table III). When the extract was treated with trichloroacetic acid the protein-free filtrate, after removal of the trichloroacetic acid with ether, was devoid of activity. The activity of the extract was unchanged after 18 hours' dialysis against running water (Table IV). In some experiments a portion of the extract was half saturated with ammonium sulphate (Mills, 1921), and allowed to stand overnight at 0° C. The precipitated protein was separated by centrifugation and made up to its original volume with 0·9 per cent. NaCl. Traces of ammonium sulphate were removed by dialysis against 0·9 per cent NaCl and the activity of the final suspension determined. It was found that the thromboplastic activity of the original extract was retained in this way (Table V). Mills (1921) has reported similar findings with lung extracts.

TABLE IV.—*The Effect of Dialysis on the Activity of 0·9 per cent NaCl Extracts of Normal and Ischaemic Muscle Extracts.*

Rat No.	Extract.	C.T. of recalcified oxalate plasma. Without extract.	With extract. Before dialysis.	With extract. After dialysis.
		2′ 14″		
45–46	Normal		23·5″	22″
	Ischaemic		21″	20·5″
47–48	Normal		27″	22·5″
	Ischaemic		19·5″	19·5″

TABLE V.—*A Comparison of the Activities of 0·9 per cent NaCl Extracts of Normal and Ischaemic Muscle and the Ammonium Sulphate Precipitated Fractions of these Extracts in the Same Concentration.*

Rat No.	Extract.	C.T. of recalcified oxalate plasma after addition of— 0·9 per cent NaCl extract.	$(NH_4)_2SO_4$ precipitated fraction.
	Saline control	1′ 47″	..
74–75	Normal	30″	45″
	Ischaemic	20″	27″

Mills (1921) and Quick (1935), studying the effect of lipoid solvents on thromboplastin preparations from lung and brain, found that, with the exception of acetone, they reduced the activity of the thromboplastin. This was confirmed for the muscle thromboplastin used in these experiments. Acetone preparations of muscle were made according to Quick's method (1942) for the preparation of thromboplastin from brain. Such preparations were active, and saline extracts of acetone-dried ischaemic muscle were more active than those of acetone dried

normal muscle (Table VI). However, neither of these preparations was as active as simple saline extracts (Table VII). In this respect muscle thromboplastin differed from that of brain, for the degree of loss of activity in the acetone preparations from muscle was always considerably greater than that in the brain preparation.

TABLE VI.—*The Effect of 0·9 per cent NaCl Extracts of Acetone Dried Normal and Ischaemic Muscle on the Coagulation of Recalcified Oxalate Rabbit Plasma.*

Rat No.	Extract.	Coagulation time.
	Saline control	4′ 10″
23	Normal	3′ 28·5″
	Ischaemic	1′ 7·5″
	Saline control	1′ 56″
29–30	Normal	1′ 19·5″
	Ischaemic	35·5″
31–32	Normal	1′ 38·5″
	Ischaemic	43·5″
	Saline control	1′ 23·5″
33–34	Normal	1′ 5·5″
	Ischaemic	59·5″
35–36	Normal	1′ 23″
	Ischaemic	46″
	Saline control	2′ 11″
54–55	Normal	42″
	Ischaemic	31″
	Saline control	2′ 8″
56–57	Normal	1′ 11·5″
	Ischaemic	36″

TABLE VII.—*A Comparison of the Effect of 0·9 per cent NaCl Extracts and 0·9 per cent NaCl Extracts of Acetone Dried Muscle and Brain on the Coagulation of Recalcified Oxalate Plasma.*

Rat No.	Tissue.	Coagulation time.	
		0·9 per cent NaCl extract.	0·9 per cent NaCl extract of acetone dried tissue.
106	Muscle (right leg)	20″	..
	,, (left leg)	..	1′ 2″
	Brain	19·5″	15·5″

Normal C.T. of plasma, 3′ 7″.

The activity of a saline muscle extract did not alter in the first 24 hours after preparation if kept at room temperature, and appeared to be stable at 0° C. Its activity was greatly reduced by heating for 15 min. in a water bath at 50° C.

and was destroyed by exposure to 60° C. for a further 15 min. (Fig. 4), in agreement with the results of Fischer (1931), who found that muscle thromboplastin was destroyed at 56° C. The same degree of thermolability was shown by the extracts after dialysis, by the acetone dried fractions and by the fractions precipitated by ammonium sulphate (Fig. 4). In this respect the crude muscle thromboplastin preparation resembles that of lung (Mills, 1921), being more thermolabile than a similar preparation of brain thromboplastin, which is not materially affected until 60° C. (Quick, 1935).

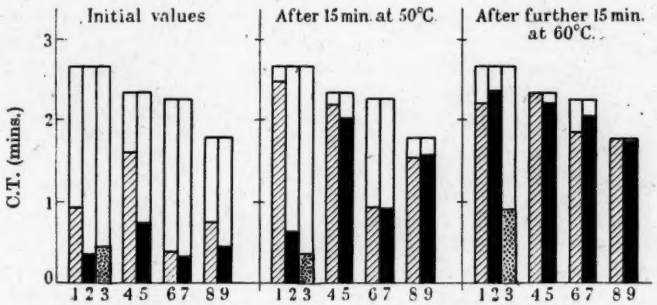

FIG. 4.—The effect of temperature on the activity of extracts from normal and ischaemic muscle and from brain. The total height of each column gives the control C.T. of the plasma, the shaded area the C.T. after the addition of the extract.

Column 1 = 0·9 per cent NaCl extract of normal muscle } Rat Nos. 26–27.
" 2 = " " " ischaemic muscle }
" 3 = " " " acetone prepared rat brain.
" 4 = " " " " " normal muscle } Rat Nos. 31–32.
" 5 = " " " " " ischaemic muscle }
" 6 = " " " normal muscle after dialysis } Rat Nos. 45–46.
" 7 = " " " ischaemic muscle after dialysis }
" 8 = " " suspension of $(NH_4)_2SO_4$ precipitated fraction of normal muscle extract } Rat Nos. 74–75.
" 9 = " " $(NH_4)_2SO_4$ precipitated fraction of ischaemic muscle extract }

Properties of the clotting factor of ischaemic muscle.

Tables I, II, IV to VI show that the properties of the clotting factor in ischaemic muscle extracts closely resemble those of the factor in normal muscle extracts. Ischaemic muscle extracts occasionally clotted oxalate plasma. This may have been due to small amounts of thrombin, but the C.T. in such cases was always so long that the amounts of thrombin present must have been minute, and would not explain the greater activity of the extracts. The only difference between the normal and ischaemic extracts, apart from the greater activity of the latter, was the greater thermostability of the ischaemic muscle extracts (Fig. 4). In this respect it more closely resembled brain thromboplastin. However, this thermostability was not a property of the thromboplastin itself, but a property conferred upon it by a dialysable constituent of the extract (Fig. 4); nor was it shown by the acetone preparations of ischaemic muscle or ammonium sulphate precipitated fractions (Fig. 4). The identity of the substance(s) concerned was not investigated.

Further investigation of the activity of ischaemic muscle extract.

The preceding experiments have shown that the clotting factor present in saline extracts of normal and ischaemic muscle behaves like a thromboplastin with similar properties to those described for the thromboplastin of lung and brain. An explanation for the greater activity of the extracts prepared from ischaemic muscle is required. The fact that this greater activity is retained after dialysis, and is present in the ammonium sulphate precipitated fraction and in the acetone preparation, suggests that the greater activity might be due to the presence of larger amounts of thromboplastin in these extracts, and hence in the damaged muscle. However, this view cannot be accepted without further enquiry. An attempt to test the theory by determining the phospholipin P content of the muscle from normal and ischaemic limbs showed that ischaemia did not alter this fraction of the muscle constituents. Possible explanations of the effect not dependent upon an absolute increase in the thromboplastin may be tabulated as follows :

(1) Contamination of the extracts by blood.
(2) pH differences between the extracts.
(3) Better extraction of thromboplastin from the damaged tissue.
(4) Presence of trypsin-like enzymes in the extracts.
(5) Activation of existing amounts of thromboplastin.

These alternatives have been investigated with the following results :

(1) Contamination of the ischaemic muscle extracts by blood might explain their increased effect on C.T. The extracts from the ischaemic muscle were frequently pinker than those from normal muscle, but their effect on C.T. could not be correlated with their colour. Table I shows that perfusion of the hind limbs did not diminish the difference between the two extracts. However, perfusion is not the answer to this problem, since much of the blood in the clamped limb cannot be removed in that way. Estimations of the total iron in the extracts were made, but no clear correlation between this fraction and the effect on C.T. was observed. The clearest demonstration of the inability of blood contamination to account for the greater activity of the ischaemic muscle extracts is provided by Quastel and Racker (1941). They found the thromboplastic activity of clear centrifugates of the medium in which tissues had been incubated anaerobically to be greater than of those after aerobic incubation, and under such conditions blood contamination was excluded.

(2) The effect cannot be explained by pH differences, since the differences between the pH of the saline extracts of normal and ischaemic muscle were only slight, and the effect was still observed when the extracts were made with M/30 phosphate buffer (pH 7).

(3) Quastel and Racker (1941) suggested that muscle ischaemia might make the contained thromboplastin more easily extractable. As a preliminary experiment portions of a muscle specimen were extracted by methods of varying efficiency. The effect of these extracts on the plasma C.T. were compared with their total and phospholipin P contents, and it was found (Table VIII) that with increasing efficiency of extraction the thromboplastin concentration and the phosphate and protein N fractions increased. Applying this result to the problem in hand, the thromboplastin content of the extracts from normal and ischaemic muscle were compared with their total and phospholipin P contents (Table IX). No correlation was seen, and the two extracts contained approximately equal

TABLE VIII.—*Effect of Different Methods of Muscle Extraction* (1 : 2 0·9 *per cent NaCl*) *on the Thromboplastin, Total and Phospholipin P and Total Protein N Contents of the Extracts.*

Method of extraction.	Thrombo-plastin content, units/ml.	Total P content, mg./ml.	Phospholipin P content, mg./ml.	Total Protein N content, mg./ml.
Muscle soaked in 0·9 per cent NaCl for 15′ and pressed with pestle	4	0·125	tr.	0·19
Muscle coarsely divided and lightly ground with pestle	24	0·454	tr.	1·40
Muscle finely divided and ground with quartz	237	0·746	0·03	2·42

amounts of total and phospholipin P. Similarly the two extracts contained equal amounts of total protein (Table IX), so that judged by these criteria the efficiency of extraction appeared to be the same for the two types of muscle.

(4) Beloff and Peters (1945) have found that trauma (heat) to the skin leads to the liberation of a proteolytic enzyme resembling trypsin. The liberation of

TABLE IX.—*A Comparison of the Thromboplastin, Total P, Phospholipin P and Total Protein N Contents of* 0·9 *per cent NaCl Extracts of Normal and Ischaemic Muscle.*

Rat No.	Extract.	Thrombo-plastin content, units/ml.	Total P content, mg./ml.	Phospholipin P content, mg./ml.	Total protein N, content mg./ml.
84–85	Normal	177	0·62		
	Ischaemic	200	0·56		
86–87	Normal	133	0·56		
	Ischaemic	422	0·51		
88–89	Normal	94	0·46		
	Ischaemic	422	0·52		
90–91	Normal	299	0·55		
	Ischaemic	398	0·55		
95–96	Normal	94	0·48		
	Ischaemic	355	0·54		
97–98	Normal	159	0·58		
	Ischaemic	501	0·54		
188–189	Normal	177	..	0·02	
	Ischaemic	501	..	0·02	
190–191	Normal	177	..	0·03	
	Ischaemic	266	..	0·04	
186–187	Normal	266	..	0·02	
	Ischaemic	562	..	0·02	
199	Normal	100	..	..	2·43
	Ischaemic	562	..	..	2·47
200	Normal	133	..	..	2·90
	Ischaemic	562	..	..	2·66

such an enzyme might be a general feature of trauma, and its presence in the extracts from ischaemic muscle might account for their greater activity, since trypsin has a coagulant action (Douglas and Colebrook, 1916). The finding that the active substance of our experiments acts on prothrombin and is activated by Ca^{++} does not exclude the participation of a tryptic-like enzyme, since these are also features of trypsin action (Eagle and Harris, 1937 ; Ferguson and Erickson, 1939). Furthermore, proteolytic enzymes have been found in lung extracts by Izumi, Tanaka and Takano (1937) and by Macfarlane and Pilling (1946). However, attempts to demonstrate the presence of such enzymes by incubating the extracts with 10 per cent casein solution proved unsuccessful. The relative crudeness of this method led to further experiments in which the effect of the extracts on the fibrin clots formed in plasma and fibrinogen solutions was observed. In the ordinary tests with undiluted rabbit plasma no fibrinolysis was seen after 24 hours' incubation. Since this result might have been due to the high concentration of anti-enzyme in rabbit blood, tests were also carried out on dilutions of rat and human plasma and on fibrinogen solutions, using both saline muscle extracts and their ammonium sulphate precipitated fractions. Definite fibrinolysis was not seen in these experiments, and the only difference between the extracts from normal and ischaemic muscle was that with the latter the clot appeared to retract more rapidly and to a greater degree.

(5) The discussion of possible enzymic mechanisms leads to the possibility that, whilst the amount of thromboplastin in the two extracts might be the same, that in th. extract from ischaemic muscle might have been activated in some way. Certain types of thromboplastin (Russell Viper Venom) are activated by the addition of commercial lecithin (Trevan and Macfarlane, 1936). The activity of saline extracts from normal and ischaemic muscle could not be increased by the addition of lecithin, although the specimen of lecithin used increased the activity of Russell Viper Venom.

During the coagulation of plasma an autocatalytic reaction is thought to occur with the accelerated production of thrombin. The mechanism of this reaction, while not fully understood, is thought (Astrup, 1944) to be due to the activation of thromboplastin, and Laki (1943) claims to have isolated such an activator (plasmakinin) from the plasma. According to him it may be differentiated from thromboplastin by its reaction with recalcified Mellanby-fibrinogen. For the action of thromboplastin it is immaterial how long the Mellanby-fibrinogen has been recalcified, but with plasmakinin the longer the Mellanby-fibrinogen has been recalcified the shorter the C.T. Such an experiment was performed with saline extracts of normal and ischaemic muscle, but no plasmakinin effect was observed.

DISCUSSION.

The results described here provide complete confirmation of the work of Quastel and Racker (1941), and give additional data for the view that the clotting factor in extracts of normal and ischaemic muscle is a thromboplastin not greatly different in its physical properties from that derived from other tissues. The only physical difference between the thromboplastin obtained from the two types of muscle is the greater thermostability of that from the ischaemic limb and, as we have seen, this is a property conferred upon it by some dialysable constituent of the extract. A further interesting feature is the decreased activity of the acetone preparations ; in this respect it resembles lung thromboplastin

(Astrup and Darling, 1942). This may be due to the removal, by the acetone, of the lipid factor, without which thrombokinase is relatively inactive. In brain, where the acetone preparations are active, there may be such an excess of the lipid factor that it is not completely removed by the acetone. This loss of activity on acetone treatment probably explains the difference in toxicity between the fresh and acetone-dried muscle extract in the preparation of Dyckerhoff myotoxin (Dyckerhoff, Schorcher and Torres, 1939; Green and Stoner, 1947).

Anoxaemia superficially appears to increase the thromboplastin content of muscle, but this view cannot be put forward till all possible sources of error have been excluded. Experiments designed to test various alternative theories have been described. From the results obtained pH differences and blood contamination can be excluded as possible factors in the increased activity of the ischaemic muscle extracts. Nor, in view of the work of Crane and Sandford (1937), can the effect be ascribed to variations in the Ca^{++} content of the extracts. The increased activity of the ischaemic muscle extracts is not due to nervous influences, since it was seen in rats in which the femoral and sciatic nerves to the clamped limb had been divided two days previously.

Since fibrinolysis is associated with trauma (Macfarlane and Biggs, 1946), the occurrence of proteolytic enzymes in the ischaemic muscle extracts seemed a strong theoretical possibility, yet there was no experimental evidence in favour of this view. Our results are in accordance with those of Chargaff, Bendich and Cohen (1944), who found that pure thromboplastin possessed no proteolytic activity, and the similar results of Dyckerhoff and Marx (quoted by Chargaff, 1945) with crude preparations. According to Seegers (1940), fibrinolysis is due to a separate enzyme. However, it is difficult to exonerate proteolytic enzymes from all complicity in the effect. Tagnon (1942) has shown that thromboplastin inhibits fibrinolysis, so that such enzymes might still be present in our extracts. Furthermore Ferguson and Erickson (1939) have shown that minute amounts of trypsin appear to activate thromboplastin. These amounts might be too small or too easily inhibited to be detected by the tests used. Moreover, although these proteolytic enzymes might be present in the extracts, they might not exert a direct coagulative effect in the clotting system, rather acting before the preparation of the extract in liberating active thromboplastin from a store of inactive "combined thromboplastin" in the cells. Certainly the more recognized forms of thromboplastin activation do not appear to be responsible. While the experiments described above, under (3), exclude any general increase in the efficiency of extraction in the case of the damaged muscle, they are not sufficiently sensitive to exclude a mechanism of this type. The problem can only be solved by further experiment, possibly involving isolation of the thromboplastin.

Further work is also necessary on a related problem. It has been shown above that although extracts from ischaemic muscle had a greater thromboplastic activity than those from the corresponding normal muscle, no increase was seen in extracts from muscle which had been allowed to autolyse for a similar period, yet Quastel and Racker (1941) had shown an increase on incubating muscle anaerobically *in vitro*. The clamp used by us does not produce complete anaerobiosis, although it almost occludes the arterial blood supply to the muscle. It may be that the reaction to partial ischaemia, as in the clamped limb, is different from that to the complete ischaemia of the autolysing limb. Results from other experiments carried out in this laboratory suggest that this may be so. There are certainly macroscopical differences between the muscle of the clamped limb

and muscle after a similar period of autolysis, the former being much more friable and "mushy." The conditions of the *in vitro* experiments of Quastel and Racker may perhaps be more closely related to those of the clamped limb than of the autolytic limb.

These changes in the ischaemic limb must also be correlated with the general changes which occur in the coagulative mechanisms of the body after such procedures (Irish, Hamilton, Haist and Jacques, 1945). It is not yet possible to describe in detail the changes which occur in the limb on the restoration of the circulation. Preliminary results indicate that the release of the clamp is followed by the formation of thrombin, with a subsequent gradual decline in the clot-promoting activity of the extracts until, after 44 hours, the activity of the two extracts is the same. Both from the changes in the general coagulative mechanisms which are known to occur in "shock" (Mylon, Winternitz, Katzenstein and de Sütö-Nagy, 1942; Irish *et al.*, 1945), and the effect of clot-promoting substances on toxic compounds possibly liberated by tissue trauma (Green and Stoner, 1947), the findings reported here would seem to have a definite bearing on the general "shock" problem, as was envisaged by Quastel and Racker.

SUMMARY.

The observations of Quastel and Racker (1941) that ischaemia of muscle increases the clot-promoting activity of 0·9 per cent NaCl extracts made from it have been confirmed.

It has been shown that the clot-promoting activity of these extracts is due to thromboplastin, and the properties of these crude extracts have been described.

The possible explanations of the greater activity of the ischaemic muscle extracts have been discussed, and it is thought that ischaemia leads to the mobilization of thromboplastin stored in an inactive form in the cells.

It is thought that these findings have a bearing on the general bodily reaction to trauma.

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IMMUNIZATION OF HUMAN BEINGS WITH RED CELL ELUATE AND OTHER TYPES OF INFLUENZA VACCINE.

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THE red cell eluate vaccine described by Francis and Salk (1942) shows some promise as a means of protection against influenza A and B. In previous investigations (Salk, Menke and Francis, 1945; Salk, Pearson, Brown and Francis, 1944; Francis, Salk, Pearson and Brown, 1944) one dose of 1 c.c. has been given. Because the virus is concentrated ten times in the process of manufacture, and little more than 6 to 9 c.c. of allantoic fluid can be collected from each egg, it follows that it takes two to three eggs to produce the virus required for one dose. This renders the vaccine very expensive. The high virus content, moreover, tends to produce reactions of varying degrees of severity in a high proportion of those immunized. Nevertheless, the dosage employed appears to have been chosen for no very definite reason beyond the fact that it was known that a greater immune response was obtainable if the virus present in raw allantoic

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fluid was concentrated (Hirst Rickard and Whitman (1942); Hirst and Horsfall, 1942; Hare, Morgan, Jackson and Stamatis, 1943). The present paper is an account of experiments carried out to determine whether as much as 1 c.c. per dose of the red cell eluate vaccine is in fact necessary, and whether smaller doses or a lower degree of concentration would not suffice.

For this purpose a red cell eluate vaccine (Lot 44–1) was chosen, containing approximately equal quantities of PR8 and LEE concentrates produced by commercial methods in these laboratories. Dilutions were made in saline and groups of 20 human volunteers were immunized. Sera taken before, and at intervals thereafter, were titrated by the red cell inhibition method in order to assess the degree of immunity produced. Two other vaccines were also employed, one made by the adsorption of influenza A on alum without concentration, and another in which the influenza A component was concentrated by freezing, thawing and elution into isinglass solution. The B component of both these vaccines consisted of red cell eluate, added immediately before immunization.

EXPERIMENTAL.

Preparation of the red cell eluate vaccine.

The vaccine was prepared as described by Hare, Curl and McClelland (1946), and consisted of 1510 c.c. of PR8 suspension and 1555 c.c. of LEE suspension. The PR8 component was made by combining four bottles harvested on Jan. 29, 1945, whose red cell agglutinating titres were 1280, 2560, 1900, 2560, and another four harvested on Jan. 30 with titres of 1280, 2560, 2560 and 2560. Their mean titre weighted by volume, calculated by the method described by Hare and Mackenzie (1947), was 2071. The LEE component was made in almost exactly the same way, except for the fact that it was a 13·3 times concentrate of 8 bottles harvested on Jan. 22 and 3 on Jan. 23, the weighted mean titre being 2560.

Antigenic tests in mice.

These were carried out by the method described by Hare and Mackenzie (1947), the vaccine being first diluted one in five for immunization against PR8, and one in five hundred against LEE. Two doses 7 days apart of 0·5 c.c. were given intraperitoneally and 7 days later living virus was given intranasally, deaths being recorded up to the 14th day.

Toxicity tests.

Two guinea-pigs survived 10 days following the administration of 5 c.c. of the vaccine subcutaneously and two mice following 0·5 c.c. intraperitoneally. Six mice showed no lesions when 0·05 c.c. was administered intranasally.

Agglutinin inhibition tests.

These were carried out by the method of Hirst and Pickels (1942), using a Klett Summerson photo-electric cell to determine the end point. Chicken red cells obtained from the slaughterhouse were employed in 1·5 per cent suspension, which was then adjusted by the addition of more cells or saline to give, after fourfold dilution, a reading of approximately 300 on the photo-electric cell. The same suspensions of PR8 and LEE viruses were used throughout, large pools

of allantoic fluid infected with these viruses having been collected, distributed into screw-capped bottles and stored in the refrigerator without freezing. The virus was titrated before use each day and then diluted so that it had a titre of 1/16, or a final titre, in the inhibition tests, of 1/4. The sera were inactivated at 55° C. for 30 minutes as soon after collection as possible. All the sera collected from each individual were, of course, titrated at the same time but, as an added precaution against errors due to variations in virus content of the suspensions employed from day to day, the sera of an equal number of volunteers from each of the vaccinated groups were titrated in each day's run. In addition, a control serum was included in every series of tests and, if its titre appeared to be abnormal, the tests were repeated.

Alum precipitation.

Sterile allantoic fluid containing influenza A virus was taken and 1/500 formalin added. To 88 g. of the formolized fluid in a sterile bottle, 7·1 c.c. sterile 2·5 per cent sodium bicarbonate solution was added and then, slowly, 10·6 c.c. sterile 5 per cent potassium alum. The precipitate which formed was allowed to settle overnight; the supernatant was then removed, and the precipitate washed twice with sterile saline. It was then resuspended in 88 g. of sterile saline. The titre of the original fluid was 1/250 and of the vaccine, after solution in 20 per cent sodium citrate solution, was also 1/250. An equal quantity of influenza B red cell eluate vaccine was added immediately before the suspension was used for immunization.

Concentration by freezing and slow thawing.

Sterile influenza A fluid was taken and 210 g. weighed out into a sterile centrifuge bottle. It was frozen in a mixture of ice and salt overnight and allowed to thaw slowly at room temperature. It was centrifuged slowly to pack the precipitate. The supernatant was then removed. To the precipitate was added 5·25 c.c. of 0·6 per cent isinglass solution containing 1/500 formalin and the mixture kept in the refrigerator overnight. The following morning 15·75 c.c. of 0·6 per cent isinglass without formalin was added and the whole allowed to reach 37° C. for 60 minutes. The bulk of the precipitate went into solution. The tube was then centrifuged and the supernatant removed. After standing in the refrigerator overnight a copious precipitate of urates appeared. The titre of the original fluid was 1/512 and of the vaccine, after solution in 20 per cent sodium citrate, 1/2560. Before use an equal amount of influenza B red cell eluate vaccine was added.

RESULTS.

The red cell eluate vaccine selected for study consisted of a mixture of nearly equal amounts of PR8 and LEE suspensions, the degree of concentration of the virus in the process of manufacture having been ten times for the PR8 component and 13·3 times for the LEE. The weighted geometric mean red cell titre of the PR8 component was 2071 and of the LEE, 2560. Vaccines equal in potency were produced in large quantities over a six-month period in these laboratories. Dilutions in sterile saline of the mixed vaccine to one-half, one-fifth and one-eighth were then made. One c.c. quantities of these dilutions as well as undiluted

vaccine were given subcutaneously in the upper arm to groups of 20 volunteers resident in Toronto on either Dec. 19 or 20, 1945. In addition to these groups, another group of 20 persons received 1 c.c. of saline.

A proportion of those receiving the vaccines suffered from varying degrees of local and generalized reaction. The incidence of these reactions is given in Table I. None of the reactions were of an anaphylactic nature such as might be expected from egg protein in a sensitive individual. The generalized reactions appeared about 6 to 8 hours following injection, and comprised fever, chills, headache and sleeplessness, lasting up to 36 hours. Local reactions without systemic reaction were also observed, and comprised varying degrees of swelling and redness of the arm.

TABLE I.—*Incidence of Local and General Reactions Following Administration of the Vaccines.*

Vaccine.	Number of persons.	Number with—		
		Fever.	Chills without fever.	Local reaction only.
Lot 44–1 undiluted	20	4	3	7
	16	5	6	3
Diluted one in two	20	0	0	0
Diluted one in five	20	2	1	0
Diluted one in eight	20	0	0	0
Influenza A adsorbed on alum + red cell eluate influenza B	20	4	5	6
Influenza A concentrated by freezing and thawing + red cell eluate influenza B	20	8	10	4
Saline	20	0	0	0

Blood was taken from all the volunteers before the injections and again after 2 weeks, 6 weeks, 10 weeks and 16 weeks. The sera after inactivation were all titrated by means of the agglutinin inhibition method against both PR8 and LEE viruses. The geometric means of the titres at each bleeding were then determined and the results plotted in the form of the graphs in Fig. 1.

The highest point reached in all but one of the curves was at the second week after immunization, the general tendency thereafter being downwards. It is at once apparent that the vaccine diluted with an equal amount of saline was only slightly less efficient than the undiluted material, greater degrees of dilution however, giving a much less efficient antigen. It will also be observed that there was a slight rise during the first two weeks on the part of individuals who had received only saline. No explanation can be offered for this other than the fact that many sera in the initial bleedings were haemolysed. Insufficient bleedings were carried out to determine the actual day on which the summit was reached, but the behaviour of the curve during the first two weeks may be judged from another series of observations in which a group of 16 normal persons received 1 c.c. doses of the undiluted vaccine on Jan. 8, 1946, and were bled at 48 hours,

96 hours, 144 hours, 14 days and 6 weeks. The titrations with these sera are given in Fig. 2, and show that with undiluted material at least there is a marked negative phase during the few days immediately following the injection. By the end of the first week the curve has usually reached the level from which it started

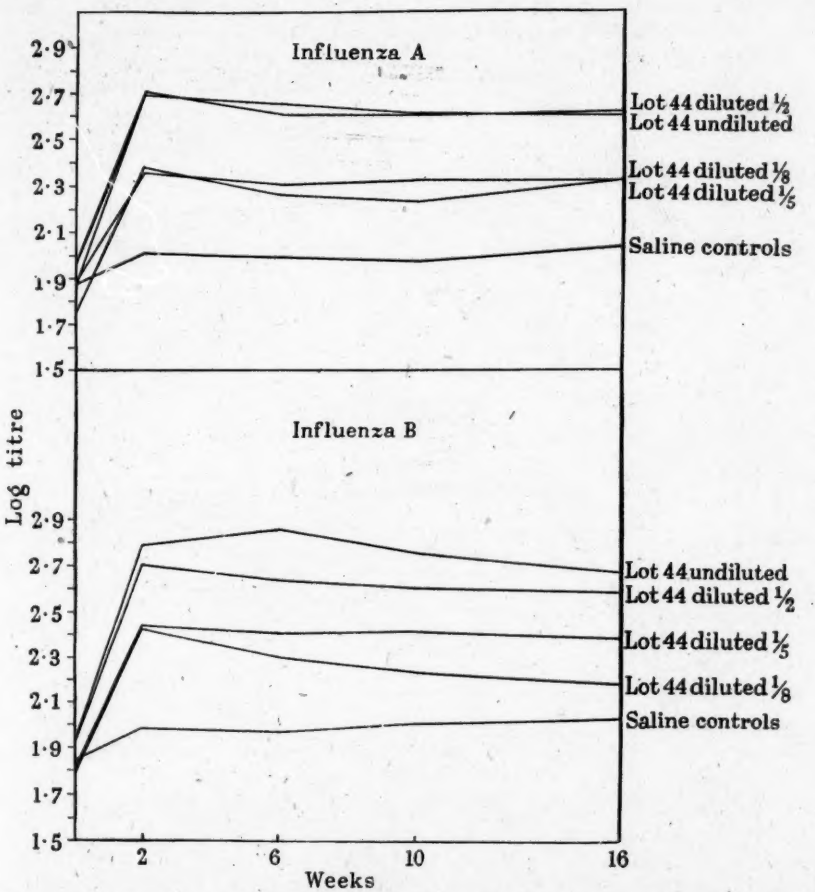

FIG. 1.—Geometric mean red cell inhibition titres of groups of 20 persons who had received one dose of saline or Lot 44–1 red cell eluate vaccine in various dilutions.

and rises steeply thereafter. No observations were made on the events immediately following injection of diluted vaccine.

In Table II are given the highest and lowest titres obtained at the bleeding two weeks after immunization, and show that even with undiluted vaccine some individuals reacted very slightly. It is also of interest that the highest titres recorded were obtained in persons who received vaccine diluted one in two.

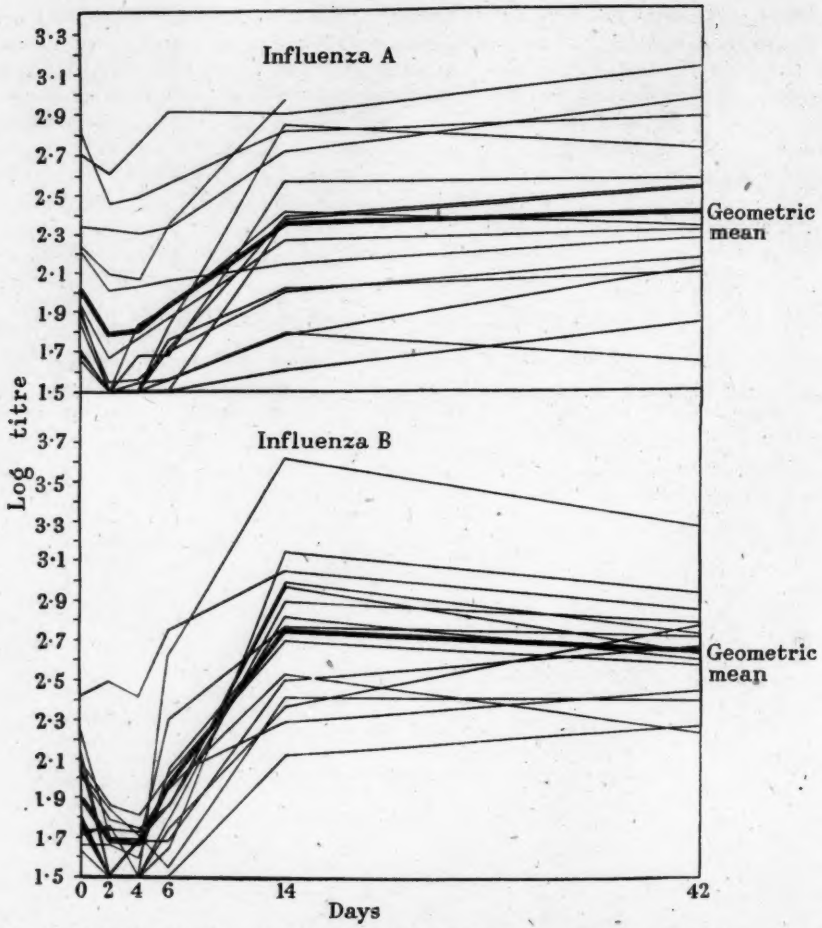

FIG. 2.—Individual and geometric mean red cell inhibition titres of 16 persons who had received one dose of undiluted Lot 44–1 red cell eluate vaccine.

TABLE II.—*Highest and Lowest Titres at Two Weeks, following One Dose of Vaccine.*

Vaccine.	Influenza A.		Influenza B.	
Lot 44–1				
Undiluted	>32/82	>32/1531	94/190	180/2755
Diluted one in two	62/117	114/7161	59/123	62/3041
Diluted one in five	32/64	603/1084	60/74	222/794
Diluted one in eight	>32/45	244/690	>32/85	283/2831

The numerators indicate the titre before immunization and the denominators the titre at two weeks.

Subsequent behaviour of the immunized and control groups.

During the period following the first two weeks there was a much greater tendency for the titre to remain high than had been expected. This was particularly the case with the antibody for influenza A. Indeed, the titres actually showed a tendency to rise during the six weeks' period between Feb. 28 and April 10. This tendency was also observed in the control group. It is probable that both the persistently high level and the tendency to an actual increase in the mean titre was due to the presence of both influenza A and B viruses in the neighbourhood during the period following immunization.

TABLE III.—*Evidence of Infection with either Influenza A or B Viruses during the Post-immunization Period.*

Name.	Influenza A.					Influenza B.					Upper respiratory infection.	
	Dec. 19 or 20.	Jan. 2 or 3.	Jan. 30 or 31.	Feb. 27 or 28.	Apr. 10 or 11.	Dec. 19 or 20	Jan. 2 or 3.	Jan. 30 or 31.	Feb. 27 or 28.	Apr. 10 or 11.	Febrile.	Non-febrile.
Saline Controls.												
RIC	97	176	167	196	186	55	64	86	228	439	..	Jan. 28
HAY	54	87	70	64	53	38	51	52	240	252	..	Feb. 2
LEE	57	95	74	83	161	80	178	127	120	202	..	Feb. 13
LAN	79	73	66	179	225	67	106	61	76	64	..	Feb. 17
Lot 44–1 undiluted.												
WIS	>32	82	46	107	75	75	208	221	176	122	Feb. 13	
Lot 44–1 diluted one in two.												
CAM	58	193	122	226	661	56	226	200	164	182		
KEL	124	451	484	991	982	42	572	326	353	272		
WAL	111	1,303	1,432	902	1,892	355	582	665	412	502		
Lot 44–1 diluted one in five.												
DIC	206	490	296	327	256	54	285	170	334	427		
RUT	>32	85	58	172	153	139	178	123	217	157		
Lot 44–1 diluted one in eight.												
COO	112	427	232	194	537	94	142	133	113	115		
McL	>32	45	>32	>32	172	46	135	116	106	94		

The figures given are the reciprocals of the titres obtained at each bleeding. Where no date is given in the last two columns there was no history of infection.

Examination of the titres of the control group for instance shows that some persons had evidently been infected with influenza A or B during the period in question. Instances in which the titre rose two or more times between bleedings are given in Table III. Two persons, RIC and HAY, were known to have suffered from non-febrile infections on Jan. 28 and Feb. 2, and examination of the titres of the appropriate sera indicates that there had been an increase of sufficient magnitude to suggest infection with influenza B. Another, LEE, also showed a similar tendency for the antibody level for influenza B to increase; this may have been a consequence of the infection on Feb. 13 or contact with febrile infections in other members of the family. A fourth, LAN, who was ill on Feb. 17, also showed evidence of a marked rise in antibody level, but in this case for influenza A. There is thus high probability that both influenza A and B were present in the Toronto area during February, 1946, and by way of confirmatory

evidence, it may be added that the presence of these two viruses was also detected in a military camp in the vicinity during the same period.

Study of the individual curves in the groups who had received the various types of vaccine showed that infections also occurred amongst these individuals. In a number, the antibody level duly increased over the initial two weeks following immunization, began to fall, and then showed evidence of a greater or lesser degree of secondary rise at subsequent bleedings. Several instances of this are given in Table III. One of the most interesting was an individual, WIS, who had received vaccine in full dosage, but who reacted so poorly that his titre for influenza A at the second week was only 82, after which it fell to 46 on Jan. 30. On Feb. 13 he suffered from what appeared to be clinical influenza with fever (100·3° F.), his titre at the subsequent bleeding being 107. Fourfold or greater increases in titre were actually observed for influenza A in two persons, one of whom, MCL had received vaccine diluted one in eight and the other, CAM, vaccine diluted one in two. These increases occurred during the period between Feb. 27 or 28 and April 10 or 11, and with both there was no evidence of clinical infection. Of the remainder, two, KEL and WAL, who had received vaccine diluted one in two, RUT who had had vaccine diluted one in five, and COO who had had vaccine diluted one in eight, showed evidence of a secondary rise for influenza A antibody, while DIC, who had had vaccine diluted one in five, showed a similar secondary increase for influenza B.

Thus the generally high level of the curves at the 16th week may well have been due to actual or subclinical infection of a number of individuals with either influenza A or B, known to have been in the neighbourhood during the period following immunization.

These observations show that the red cell eluate vaccine employed induced rises in antibody level in the majority of individuals, but that with full doses this was preceded by a period of about a week, during which there was usually a negative phase in the titres for both influenza A and B, that the amplitude of the succeeding rise varied greatly, some showing little increase, others a very considerable degree, that the general level reached was on the whole higher with increasing dosage of vaccine, that when immunization is carried out when influenza A or B viruses are present amongst the immunized population the mean antibody level showed little tendency to fall over a 16-week period, and that infection may occur (with fourfold increase in antibody level) in persons who have received submaximal doses of vaccine.

Antigenic Tests in Mice with the Vaccine and its Dilutions.

The Division of Biologics Control of the National Institute of Health, Bethesda, Md., U.S.A., laid down (third revision of April 10, 1945) certain standards for red cell eluate influenza A and B vaccines. In view of the fact that the Weiss strain was not incorporated in the vaccine employed in these experiments, these tests could not be exactly duplicated. Nevertheless the general plan was followed, and the technique employed has been described elsewhere (Hare and Mackenzie, 1947). Two doses of 0·5 c.c. of the vaccine administered intraperitoneally into mice 7 days apart were required to protect 50 per cent or more of six mice when challenged 7 days after the last dose with, in the case of PR8 virus, 10,000 fifty per cent lethal doses, and in the case of LEE virus, 1000 fifty per cent lethal

doses. For mice challenged with PR8 virus, the vaccine used for immunization was diluted one in five and for LEE virus, one in 500.

An exactly similar plan was followed with Lot 44–1 employed in the experiments in this paper. In addition, however, a similar series of dilutions to those employed for the immunization of the volunteers was also made up, and each dilution treated in exactly the same manner as the undiluted material. The results are given in Table IV.

Two separate series of tests were carried out, and it is evident that even when the vaccine had been diluted one in eight, it was still capable of passing the test for influenza A, less than 50 per cent of the mice dying. The test is evidently more stringent when applied to influenza B, for while both the undiluted vaccine and that diluted one in two passed the test, those diluted one in five and one in eight failed.

TABLE IV.—*Red Cell Titres and Antigenic Tests in Mice of Lot* 44–1 *Vaccine and the Dilutions used for Immunization of Volunteers.*

Vaccine.	Influenza A (PR8).			Influenza B (Lee).		
	Red cell titre.	L.D. 50's.		Red cell titre.	L.D. 50's.	
		20,000.	9840.		1394	1000.
Lot 44–1 undiluted	2071	0/8	0/8	2560	2/8	0/8
Lot 44–1 dilution $\frac{1}{2}$	1035	0/8	0/8	1280	3/8	2/7
Lot 44–1 dilution $\frac{1}{5}$	412	0/8	0/8	512	7/8	6/8
Lot 44–1 dilution $\frac{1}{8}$	259	1/8	1/8	320	7/8	4/8
Saline control	0	8/8	8/8	0	8/8	7/8

The numerator represents specific deaths within 14 days and the denominator, number of mice infected.

These results show that an influenza A vaccine which contains so little virus that its red cell titre is in the neighbourhood of 259 is capable of passing the antigenic test, that such a vaccine produces measurably lower degrees of immunity in human beings than vaccines containing more virus and that such "immunized," persons may become infected during the post-vaccination period. The tests for potency in regard to influenza B are more stringent, but even so, the vaccine under consideration may be diluted with an equal amount of saline and pass the test, giving lower responses than undiluted vaccine in human beings.

Immunization with Alum Precipitated Influenza A Virus.

Twenty persons were immunized with 0·5 c.c. of influenza A virus precipitated on alum mixed with 0·5 c.c. of influenza B red cell eluate vaccine. Bleedings were carried out on the same days as were the groups in the preceding section. The influenza A component was not concentrated.

The results obtained are given in Fig. 3 and show that there was little or no adjuvant effect, the titre being at its highest at 14 days and falling slowly thereafter. The red cell titre of the vaccine was 256, which corresponds closely to the 259 of the red cell eluate vaccine Lot 44–1 diluted one in eight. The two weeks' post-vaccination titres do not suggest that the alum materially increased the antigenic power of the vaccine.

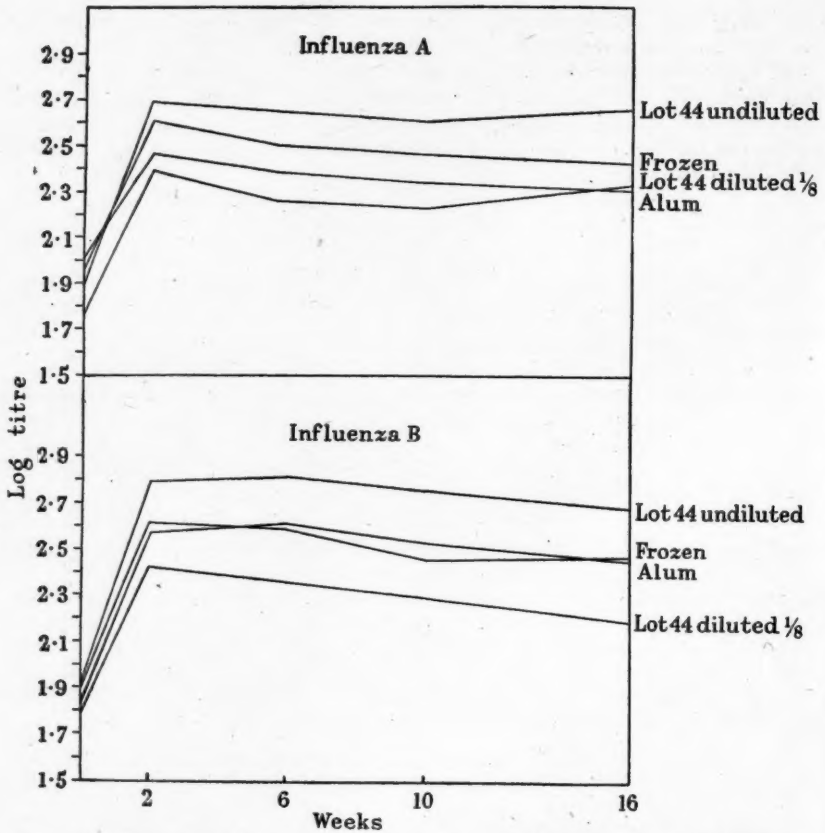

FIG. 3.—Geometric mean red cell inhibition titres of groups of 20 persons who had received one dose of influenza A virus vaccine precipitated on alum or concentrated by freezing and slow thawing, mixed with an equal quantity of red cell eluate influenza B vaccine. For purposes of comparison, the results obtained with Lot 44–1 vaccine undiluted and diluted one in eight are also included.

Immunization with Influenza A Vaccine Concentrated by Freezing and Slow Thawing.

A considerable degree of concentration of virus may be obtained if allantoic fluid is frozen and slowly thawed, the precipitate centrifuged down, the supernatant removed and the virus eluted into small amounts of buffer (Hare, McClelland and Morgan, 1942; Hirst, Rickard and Whitman, 1942). The vaccine employed in the present series of experiments was eluted into 0·6 per cent isinglass solution. Immediately after preparation, it is a faintly yellow opalescent suspension, but after standing overnight in the refrigerator a white precipitate of urates separates. It seemed possible that this precipitate might act as an adjuvant. For this reason a vaccine made in this way was mixed with an equal quantity of influenza B red cell eluate vaccine and doses of 1 c.c. administered

to 20 volunteers. They were bled at the same time as the other groups and their titrations run at the same time. The results are given in Fig. 3.

The red cell titre immediately after preparation was 2560, which corresponds closely with 2071, the mean titre of the influenza A component of the undiluted Lot 44–1 employed for immunization of the other groups. There was no obvious adjuvant action on the part of the urates, and the two weeks' post-vaccination titre was below that of the undiluted Lot 44–1 vaccine. There would therefore appear to be no particular advantage in this method of preparation.

DISCUSSION.

The experiments quoted in this paper show that on the whole the amplitude of the serological responses will depend on the amount of virus administered. Nor would there appear to be any advantage in adsorption on alum, or the presence of urates, both vaccines giving responses in conformity with the amount of virus they contained. The red cell eluate vaccine employed (which judging by the results of Hare, Curl and McClelland (1946) was a fairly typical example of a vaccine produced on a large scale) could apparently be diluted with an equal amount of saline without serious loss in immunizing potency, but if further diluted, the post-vaccination titres were markedly lower. The antigenic tests in mice showed, however, that even the weakest vaccines would pass the test for influenza A with little difficulty, but that the test for influenza B was more stringent.

Thus the results obtained in this paper suggest that the original allantoic fluid containing influenza virus must be concentrated 10 or 13·3 times, that full doses of 1 c.c. of the concentrate should be given, that a proportion of those receiving this dosage will suffer reactions, that a negative phase will be produced, and that if antigenic tests are employed as a measure of the potency of vaccines, they must be made considerably more stringent than those at present employed.

CONCLUSIONS.

1. To obtain maximum serological response, following immunization with influenza A and B eluate vaccine, it is advisable to give at least 1 c.c. of the mixed vaccine.
2. The vaccine should be concentrated 10 or 13 times.
3. Reactions will be produced in a proportion of those immunized.
4. A negative phase lasting 48 to 72 hours will probably occur.
5. The amplitude of the serological rise will vary greatly.
6. There is evidence that influenza A and B may cause sufficient infection in a proportion of those immunized to give secondary increases in titre some time after immunization.
7. Reasons are put forward indicating that the mouse antigenic test employed before issue of the vaccine is too lax as a measure of antigenic value of vaccines for the immunization of human beings.

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PLASMA AND CORPUSCULAR CHOLESTEROL IN SPRUE.

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THE most outstanding biochemical abnormality found in patients suffering from sprue is the presence of increased amounts of fat in the stools. As a result many investigations have been devoted to the determination of the degree of failure in the absorption of fatty acids and neutral fat in this condition (Thaysen and Hess, 1932, 1935; Mogensen, 1937; Black and Fourman, 1946). Since it is generally conceded that phosphorylation is an important mechanism by which fatty acids are absorbed from the intestine (Bloor, 1939; Verzár and Laszth, 1935), the theory that this mechanism is upset and is responsible for the defective absorption of fat in cases with sprue has been advocated (Stannus, 1942).

In recent years, however, cholesterol has been considered to be a possible carrier of fatty acids across the intestinal wall (Bloor, 1939; Boyd, 1937; Frazer, 1940). As long ago as 1917 Knudson reported an increase in blood cholesterol ester during the absorption of fat. Furthermore Cooke (1938) showed that cholesterol was not absorbed by individuals receiving a fat-free diet, and Brockett, Spiers and Himwich (1934) noted a marked rise in both free and combined cholesterol in the thoracic duct lymph of dogs during fat absorption.

The possibility that defective fat absorption in patients with sprue may be due to other causes than defective phosphorylation cannot be excluded. In view of this, estimations of plasma and corpuscular cholesterol, both free and in the combined state, were made in patients suffering from sprue. The estimations were made on the blood of fasting patients and while active absorption of fat was occurring. Similar observations were made upon a series of normal individuals. The results obtained in both groups are reported here. They are compared and their significance is determined by application of " Student's " t. test of comparison of the means.

DATA ON PATIENTS.

Five cases in whom an unequivocal diagnosis of sprue was established were used in this investigation. The results obtained were compared with those from a series of five normal individuals. All the patients were males. They had served in the Armed Forces in tropical countries for two or more years, and were admitted to hospital with severe symptoms of the disease. The criteria used for establishment of the diagnosis were a history of weight loss, anorexia and

flatulence, and the presence of steatorrhoea and anaemia. A summary of the relevant clinical and laboratory findings are given in Table I. During their stay in hospital all patients were given a diet containing 90 g. of fat. The amounts of protein and carbohydrate given were within the accepted daily requirements of these foods. The vitamin content of the diet was adequate.

METHOD OF INVESTIGATION.

The feeding of a fat meal which in the experience of other workers produces changes in the level of blood cholesterol offered some difficulty in cases of severe sprue suffering from diarrhoea and anorexia. Furthermore watery diarrhoea *per se* might adversely affect the absorption of fatty acids from the intestine. In order to avoid these difficulties all patients were given intramuscular injections of Hepastab (Boots). Two cubic centimetres of this extract were given daily for 5–14 days before the investigations were started. It has been found by Black and Fourman (1946) that such injections have a rapid effect in amelioration of the diarrhoea, and when given in small doses for so short a period of time have no detectable effect upon fatty acid absorption.

The procedure adopted with the normal individuals and with the patients was similar. A fast was imposed from the evening to the morning of the following day. Samples of fasting blood were obtained, and each individual was given a meal containing 18 g. of milk fat in 300 c.c. The lecithin and cholesterol contents of this meal were negligible. Subsequent samples of blood were withdrawn $2\frac{1}{2}$, 3, $3\frac{1}{2}$ and 4 hours after ingestion. Although this amount of fat is smaller than that used by most other workers, it is the same as that used by Black and Fourman. It was found by them to be easily tolerated by patients with sprue premedicated with liver extract. Experience suggested that the maximum deviation from the fasting values of blood cholesterol occurred between two and four hours after administration of the meal.

TECHNICAL PROCEDURES.

Total cholesterol of the plasma and whole blood was determined by the method of Sackett (King, 1945) with one modification: an alcohol acetone mixture (1 : 1) was used for the extraction in place of Bloor's mixture. Esterified cholesterol of plasma and whole blood was determined by the method of Bloor (1943), in which the free cholesterol is separated from the ester fraction by precipitation by digitonin. The esterified cholesterol is subsequently redissolved in petrolic ether and chloroform. The colours developed were read in a Klett-Summersen photo-electric colorimeter, using a red filter with an approximate spectral range of 640–700 millimicrons. The free and esterified content of the blood cells was calculated from the determinations made upon the whole blood and knowledge of the haematocrit.

RESULTS.

Fasting blood values.

The results obtained from determinations on the fasting bloods of both groups of individuals are shown in Table II. The values obtained for total plasma cholesterol in the normal individuals range from 182 to 223 mg. per 100 ml., with an average of 206. These figures are within the normal range established by Bloor (1916), Sperry (1936), Peters and Man (1943) and Clarke and Marney (1945).

TABLE I.—*Summary of Clinical and Biochemical Features in Five Patients Suffering from Sprue.*

Case.	Age in years.	Duration of gastro-intestinal symptoms, in months.	Tongue.	Pounds under normal weight.	Total fat content of dried stool (24 hrs.), gm. per cent.	Hb., gm. per cent.	Mean corpusc. Hb. conc. per cent.
1	28	6	Normal	22	38·0	9·0	39·0
2	28	5	Superficial erosions	34	59·2	7·0	35·0
3	25	9	Severe glossitis	48	31·4	12·8	38·4
4	43	4	Painful	28	56·0	10·0	40·0
5	39	5	,,	42	43·0	12·0	31·9

TABLE II.—*Fasting Values of Total Plasma and Corpuscular Cholesterol, Fatty Acid of the Plasma as Cholesterol Ester, and Ester/total Cholesterol Ratio in Normal Individuals and Cases of Sprue.*

Case.	Patients with sprue.							Normal individuals.							
	1	2	3	4	5	Average.	Range.	1	2	3	4	5	Average.	Range.	P.
Total cholesterol in mg. per 100 c.c. in plasma and in cells	160	127	103	148	193	146	103–193	211	182	223	222	190	206	182–223	·01
	202	232	62	173	200	174	62–232	171	195	213	290	156	205	156–290	>·05
Fatty acid as cholesterol ester in m. Eq. per litre	3·10	2·36	1·89	2·8	3·88	2·81	1·89–3·88	4·20	3·37	4·05	4·30	3·68	3·92	3·37–4·3	<·01
Fasting plasma ester/total cholesterol ratio	75	73	71	71	75	73	71–75	77	71	71	75	74	73·6	71–75	—

The values obtained from the plasma of the five patients with sprue differ significantly from the normal figures (P = ·01). The range in this group is 103 to 193 mg. per 100 ml., with an average of 146. These results confirm those obtained by Newman, Morris and Bahr in 1926 and Fairley in 1930. The total cholesterol contained in the red blood cells of both groups of individuals is not, however, significantly different. The average obtained in the series of patients is 174 mg. per 100 ml. (range 62–232), and the average obtained from the cells of normal people is 205 mg. per 100 ml. (range 156–290). MacCodom and Shiskin (1923) reported that the total corpuscular cholesterol remains within normal limits in anaemias of various types until the cell counts fall to very low levels. The ratio of esterified cholesterol to total cholesterol of the plasma is similar in both groups and is normal. The average ratio found in the series of normal individuals is 73·6 per 100 ml. (range 71–75), and the corresponding average in the series of patients is 73 per 100 ml. (range 71–75).

The fatty acid of the plasma in the form of cholesterol ester is also shown in Table II. These figures were calculated from estimations of the plasma cholesterol ester using the formula of Peters (1943), and are expressed as milli-equivalents per litre of plasma. In the normal cases this ranges from 3·37 to 4·30 m.Eq. per litre (average 3·92 m.Eq. per litre). In the cases suffering from sprue the corresponding range is 1·89 to 3·88 m.Eq. per litre with an average of 2·81 m.Eq. per litre. These figures are significantly different (P = <·01). There was no cholesterol ester found in the cells of either group of fasting individuals.

Blood curves.

The results of determinations obtained on samples of blood withdrawn 2½, 3, 3½ and 4 hours after ingestion of a fat meal from normal people and from those suffering from sprue are shown in Tables V and VI. In order to facilitate comparison of results, the average change from the fasting value of the various constituents was obtained by addition of the differences between the fasting value and the four values obtained after the meal and by dividing this figure by four. These results are shown in Table III. Positive and negative signs represent a rise and fall in level of the blood constituent respectively. In the tables are shown the average increments of the following blood constituents : total plasma cholesterol, total plasma cholesterol ester, total free plasma cholesterol, total corpuscular cholesterol per 100 c.c. of cells and total cholesterol ester per 100 c.c. of cells.

Total plasma cholesterol.

In the five patients suffering from sprue a fall in the total plasma cholesterol was observed following the fat meal. This ranges from 6·75 mg. to 12·75 mg. per 100 ml., with an average of 8·35. In four of the normal individuals a rise in total plasma cholesterol occurred, and in the fifth case no change was observed. The average for the five is 10·5 mg. per 100 ml., with a range of 0–24·5. These figures are of course significantly different.

Total plasma cholesterol ester.

An increase in plasma cholesterol ester was recorded in all cases suffering from sprue. This ranges from 2·0–15·25 mg. per 100 ml., with an average of 7·05. These results do not differ significantly from the changes seen in the level

TABLE III.—*Increments of Plasma and Corpuscular Cholesterol and Cholesterol Ester in Normal Individuals and Patients with Sprue during Fat Absorption.*

Case.	Patients with sprue.						
	1	2	3	4	5	Average.	Range.
Total cholesterol per 100 plasma	−8·25	−6·75	−6·75	−7·25	−12·25	−8·35	−6·75–−12·25
Cholesterol ester per 100 plasma	+7·5	+15·25	+6·0	+4·5	+2·0	+7·05	+2·0–+15·25
Free cholesterol per 100 plasma	−15·75	−22·0	−12·75	−11·75	−14·75	−15·4	−11·75–−22·0
Total cholesterol per 100 cells	+27·0	+147·0	0	+17·5	−12·25	+35·85	0–+147·0
Cholesterol ester per 100 cells	0	+13·75	0	0	0	+2·75	0–+13·75

Normal individuals.							
1	2	3	4	5	Average.	Range.	P.
+14·5	+24·5	+6·25	+7·25	+0	+10·5	0–+24·5	<·01
+10·25	+16·5	+1·75	+3·0	+0	+6·3	0–+16·5	·9
+2·5	+8·0	+4·5	+4·25	+0	+3·85	0–+8·0	<·01
+19·2	−16·5	+23·7	−21·25	+27·6	+6·47	−21·25–+27·6	·5
+19·7	+30·5	+15·8	+4·7	+9·5	+16·04	+4·7–+30·5	<·01

TABLE IV.—*The Cholesterol Ester/total Cholesterol Ratio in Five Patients with Sprue and Five Normal Individuals Following the Ingestion of Fat Meal.*

Case.	Normal individuals.					Patients with sprue.				
	Fasting.	2½	3	3½	4 hrs.	Fasting.	2½	3	3½	4 hrs.
1 ..	77	75	79	72	73	73	76	88	87	85
2 ..	71	68	72	70	73	75	83	96	86	90
3 ..	71	70	69	69	69	73	79	77	77	83
4 ..	75	74	73	74	75	71	74	76	79	83
5 ..	74	74	74	74	74	75	75	82	88	87

of plasma cholesterol ester in the group of normal individuals. The average rise in cholesterol ester in this group is 6·3 mg. per ml., with a range of 0–16·5.

Total free plasma cholesterol.

The free cholesterol of plasma was calculated by subtracting the result obtained from the determination of the esterified form from the total cholesterol. In the cases suffering from sprue there was a marked fall in the level of free plasma cholesterol. This ranges from 11·75 mg. per ml. to 22·0 mg. per ml. (average 15·4). In four of the normal subjects a rise in free plasma cholesterol was found. The range in this group is from 0–8·0 mg. per ml. (average 3·85). These results are significantly different ($= P < \cdot 01$).

Total corpuscular cholesterol.

In both groups of subjects the total corpuscular cholesterol showed inconstant changes. As may be seen from Table III, there occurred a fall in the corpuscular cholesterol in one individual with sprue and in two normal people. A rise was observed in three of sprue and in three normal individuals. No conclusions can be drawn from these results.

Total corpuscular cholesterol ester.

As has already been stated, no cholesterol ester was detected in the cells of the normal subjects or of the fasting patients. During fat absorption the average increment observed in the normal subjects was 16·04 mg. per ml. (range 4·7–30·5), while in only one case of sprue did the ester appear in the cells and produce an increment of 13·75 mg. per ml. In the four other cases studied no ester was found in the red blood cells. In this respect the normal subjects differ significantly from those suffering from sprue ($P = < \cdot 01$).

The ester/free cholesterol ratio.

The fasting ratio of esterified cholesterol to the total cholesterol of the plasma has been shown to be similar in both groups of individuals. During fat absorption no significant change was observed in the ratio in normal individuals. The changes in the ester/total cholesterol ratio which occurred during fat absorption in the cases with sprue are shown in Table V. In all cases there is a considerable increase in the fraction of esterified cholesterol when expressed as a percentage of the total plasma cholesterol. In case No. 2 this reaches 96·6 per cent in the third hour after the ingestion of the fat meal. In all cases ratios of over 80 per cent are attained during the period of fat absorption.

DISCUSSION.

The significant differences observed in the changes of plasma and corpuscular cholesterol in normal individuals and in patients suffering from sprue are not easily interpreted. Investigations of changes in blood cholesterol during fat absorption in human subjects and in experimental animals have been made by numerous workers with contradictory results. Several investigators (Zucker, 1920; Bloor, 1916; Csonka, 1918; Bang, 1918) failed to obtain any rise in blood cholesterol with fat feeding. Knudson (1917), Terroine (1914) and Iscovesco (1912) reported rises in blood cholesterol during fat absorption. The findings

TABLE V.—*Changes in Blood Cholesterol in Five Normal Individuals following the Ingestion of Fat Meal.*

Case.	Total cholesterol of plasma in mg. per 100 ml.					Total cholesterol of cells in 100 ml. blood in mg. per 100 ml.					
	Fasting.	2½	3	3½	4 hrs.	Fasting.	2½	3	3½	4 hrs.	Haematocrit %.
1	211	222	220	232	228	82	81	82	103	99	47
2	182	202	210	210	204	96	90	87	87	87	49
3	223	227	230	230	230	88	117	97	94	83	41
4	222	227	236	229	225	119	116	102	96	90	41
5	190	190	190	190	190	74	87	93	84	84	47
	Total cholesterol ester of the plasma in mg. per 100 ml.					Total cholesterol ester in cells of 100 ml. blood in mg. per 100 ml.					
1	163	166	175	177	175	2	0	7	52	18	47
2	130	138	152	146	150	1	12	11	23	18	49
3	156	156	159	158	158	1	27	0	0	0	41
4	167	167	173	170	170	0	5	0	2	0	41
5	142	142	142	142	142	0	3	7	6	2	47

TABLE VI.—*Changes in Blood Cholesterol in Five Cases of Sprue following the Ingestion of Fat Meal.*

Case.	Total cholesterol of plasma in mg. per 100 ml.					Total cholesterol of cells in 100 ml. blood in mg. per 100 ml.					
	Fasting.	2½	3	3½	4 hrs.	Fasting.	2½	3	3½	4 hrs.	Haematocrit %.
1	160	158	146	149	154	81	79	98	102	88	40
2	127	127	116	119	119	47	73	98	90	83	20
3	103	100	99	96	90	19	11	19	21	25	31
4	148	148	145	140	130	57	57	61	64	69	33
5	193	188	185	173	175	86	81	70	86	85	43
	Total cholesterol ester of the plasma in mg. per 100 ml.					Total cholesterol ester in cells of 100 ml. blood in mg. per 100 ml.					
1	120	120	129	130·5	130·5	0	0	0	0	0	40
2	92	106	112	103	108	0	4	0	5	2	20
3	73	79	77	77	83	1	0	0	0	0	31
4	105	110	110	110	108	0	0	0	0	0	33
5	150	149	153	153	153	0	0	0	0	0	43

of these latter workers have been confirmed more recently by Bodansky (1931), and Iscovesco has suggested that the disparities are due to a neglect of the cholesterol content of the meal fed.

From the results presented a rise in total plasma cholesterol occurs in normal individuals under the conditions of this experiment. Furthermore it is clear that this rise is due to an increase of both the free and esterified forms. During the period of fat absorption and while the alterations in plasma cholesterol are occurring, the ratio of free to esterified cholesterol is maintained constant. In the five cases of sprue who were studied the increments of cholesterol ester of the plasma are essentially similar to the corresponding rises in plasma cholesterol ester in normal individuals. These increases appear to take place at the expense of free plasma cholesterol. As a result in all cases there is a fall in free plasma cholesterol which is great enough to produce a fall in total plasma cholesterol. The diverging tendencies of free and esterified plasma cholesterol result in a marked alteration of the free/ester ratio in a direction not commonly seen in disease.

The increment of cholesterol which appears in the blood during fat absorption has been presumed by most workers to originate from the free cholesterol secreted by the gall bladder. This cholesterol is partially esterified in the intestine before absorption. Burger (1928) has shown that the amount of cholesterol secreted by the gall bladder, if reabsorbed, would suffice to account for the changes in blood cholesterol during fat absorption. An alternative source of the cholesterol was suggested by Milbradt (1930). He noted a fall in the cholesterol content of the adrenal gland with the feeding of triolein. Chaffard, Laroche and Grigant (1920) consider that the adrenals do not contain sufficient cholesterol to account for the blood changes. It may be presumed, however, that the source of the cholesterol supplying that amount which results in the rise in free plasma cholesterol in the normal individual is defective in cases of sprue. As a result the system which is responsible for the maintenance of the free/ester cholesterol ratio appears to be overcome.

The changes observed in the cholesterol ester content of the red blood cells of normal individuals confirm the findings of Knudson (1917) and Bodansky (1931). It is clear, however, that this increment does not appear to the same extent in cases suffering from sprue. As a result the overall blood fatty acid increment that appears as plasma and corpuscular cholesterol ester is very much less in patients suffering from sprue than in normal individuals. Sperry and Stoyanoff (1937) have shown that haemolysed red blood cells inhibit the action of serum cholesterolesterase ; and the absence of cholesterol in red corpuscles in the fasting state suggests that their origin during fat absorption is by a permeation of preformed ester from the plasma. It is possible that the failure of the ester to enter the red cells in cases of sprue is due to an increased resistance of their membranes.

The abnormal changes in blood cholesterol during fat absorption in a disease in which fatty acid absorption is known to be defective is presumptive evidence that the two phenomena are closely related. It is conceded that there is no incontrovertible proof that the normal increment of cholesterol in the blood originates from the cholesterol secreted by the gall bladder. The evidence which is available strongly supports this view. Thoracic duct fluid has a high cholesterol ester content, and cholesterol is esterified when incubated with fat and pancreas (Mueller, 1915, 16). Assuming this to be the case, it is clear that in cases

of sprue the diminished increment of blood fatty acids as cholesterol ester must reflect either a diminution in the ester formed in the gut, or a failure of absorption of the esterified compound. In either case it may be said that the failure to absorb fatty acids in this condition may not be wholly attributed to one specific biochemical lesion of impaired phosphorylation, but may partially be the result of an impairment of the mechanism of fat transport that is mediated by bile cholesterol.

SUMMARY.

1. The fasting values of plasma and corpuscular cholesterol and cholesterol ester of five normal individuals and five patients suffering from sprue are given and compared.

2. The changes in plasma and corpuscular cholesterol and cholesterol ester consequent upon the ingestion of 18 g. of milk fat are reported in both groups of individuals.

3. The significance of the difference observed between these patients and normal individuals is discussed in relation to the suggested function of bile cholesterol as a mediator in fat transport in the gut and the established deficiency of fat absorption in patients with sprue.

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